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**CHARACTERIZATION OF A THERMOSTABLE
AMIDASE AND DEVELOPMENT OF A BIOREACTOR
PROCESS FOR LACTIC ACID PRODUCTION**

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ABSTRACT

This thesis reports studies of the biochemical properties of a newly isolated thermostable amidase from *Bacillus* sp. RAPc8, and development of a continuous reactor process for the production of a target product. The amidase was cloned and over-expressed in *E. coli* BL21 strain pNH 223 pLySs by Cameron (2001). Earlier work by Cameron (2001) on the production and purification of the recombinant amidase showed that the enzyme could be produced with high levels of expression in shake-flask culture. Furthermore, preliminary studies have also shown that the molecular weight of the amidase was approximately 35kDa, and that it acts optimally at a temperature and pH of 50°C and 7.2 respectively.

In this study, the amidase from *Bacillus* sp. RAPc8 was produced with high levels of expression in a 5L BIOFLO 110 series fermentor. Typical biomass yields of 0.5g/litre cells (wet weight) were obtained, with the specific activity of crude amidase extracts reaching approximately 20U/mg. Extraction of the cellular proteins by centrifugation and sonication resulted in crude extracts containing an average of 426mg/ml protein in 50mM phosphate buffer. Purification of the crude enzyme extracts by a single heat-treatment step at 75°C for 45 minutes resulted in the removal of up to 70% of the unwanted proteins, and gave a partially purified protein with specific activity of up to 1800U/mg.

Studies of the biochemical properties of the amidase confirmed that the optimal temperature and pH for the amidase activity are 50°C and 7.0 respectively. Furthermore, the amidase exhibited high thermal stability at 50 and 60°C, with half-lives well beyond 3hrs at both temperatures. However, at 70 and 80°C, the amidase exhibited lower thermal stability, with half-lives estimated to be approximately 45 and 8 minutes respectively. A study of the substrate- and chiral-specificity of the amidase showed that the enzyme catalyzed the hydrolysis of only short- and mid-length aliphatic amides, and was D-specific towards lactamide. Further, the enzyme catalyzed acyl transfer in the presence of hydroxylamine, leading to the formation of hydroxamic acids.

Studies on potential inhibition of the amidase by various chemical compounds and the presence of water-miscible organic co-solvents have shown that heavy metals such as Hg^{2+} , Co^{2+} and Fe^{3+} and co-solvents such as dimethyl sulfoxide, dimethyl sulfide and 2-mercapto-ethanol severely affected the activity of the amidase, while the presence alkaline metal chlorides (NaCl , NaNO_3), chelating reagents (ethylenediaminetetraacetic acid) and protease inhibitors (phenylmethylsulfonyl fluoride) did not affect the activity of the amidase. Furthermore, the presence of dithiothreitol enhanced the activity of the amidase by 150%.

Stabilization of the amidase, and development of a potential industrial biocatalyst through immobilization, were investigated. Immobilization by entrapment in a polyacrylamide gel, covalent binding on Eupergit C beads at 4°C and covalent binding on Amberlite-XAD57 resulted in low protein binding yield and immobilized amidase activity. However, immobilization by covalent binding on Eupergit C beads at 25°C with subsequent cross-linking resulted in high protein binding yield and high immobilized amidase specific activity (recovered specific activity). Immobilization by the former techniques resulted in maximum protein binding yields and immobilized activity of 35.4 and 28.2% respectively, while the latter techniques resulted in protein binding yields and immobilized activities of up to 93.6 and 81.4% respectively. Primary characterization of the amidase immobilized on Eupergit CM beads showed that both the optimum reaction temperature and pH remained unchanged, but the stability of the immobilized amidase was enhanced at high and low reaction temperature and pH, as compared with the free enzyme. Furthermore, the thermal stability of the immobilized amidase was enhanced at temperatures between 50 and 70°C . Improved thermal stability of the immobilized amidase was observed by the ability of the immobilized amidase to exhibit significant catalytic activity under pH and temperature conditions in which the free amidase exhibited lower or no catalytic activity.

The market demand for lactic acid, its recent industrial application in preparation of biodegradable polymers and the ability of the amidase to catalyze its synthesis led to it being chosen as a target product. Thus, a nitrile biotransformation method for potential

industrial production of lactic acid was proposed. The method involves firstly the synthesis of lactonitrile by a chemical method, followed by a 2-stage hydrolysis of the lactonitrile to lactic acid and ammonia, using a dual-enzyme (nitrile hydratase and amidase from *Bacillus* sp. RAPc8) system. The proposed method has advantages over the current chemical and carbohydrate fermentation methods, including potential continuous production and recycling of immobilized enzyme. Batch reaction studies for D-lactic acid production showed that high concentrations of the racemic substrate (D,L-lactamide) and the product D-lactic acid were potential inhibitors of the amidase. The Michaelis-Menten kinetic constants K_m and V_{max} were estimated to be 556.7mM and 3.97mM/min. The high value of K_m as compared to that of free enzyme, suggest that the reactions catalyzed by the immobilized amidase are diffusion limited.

A 100 cm³ laboratory-scale bioreactor, comprising a borosilicate glass column fitted with a heating/cooling jacket and a 200µm sintered glass mesh, was used to demonstrate the potential application of the amidase in an industrial process. The fixed-bed reactor process showed the conversion of D-lactamide to D-lactic acid decreased with time under the experimental conditions, and the maximum conversion achieved was just under 30%. Mathematical modeling of the reactor process resulted in a biocatalyst thermal-deactivation model that fitted the experimental data closely, suggesting that biocatalyst thermal-deactivation affects the productivity of the reactor. The model predicted that the maximum achievable conversion, under the experimental setup and reactor size used, would be approximately 50%. Therefore, the productivity of the fixed-bed process was limited by the size of the reactor, in which only a limited amount of biocatalyst could be loaded inside the reactor. Recommendations for potential improvement of this process were made, including design of a suitable size fixed-bed reactor and investigation of other reactor configurations (e.g. membrane, stirred tank and fluidized bed reactors).

In conclusion, although the fixed-bed reactor process requires further studies and optimization, the study has demonstrated that the amidase biocatalyst has potential for future application in the production of carboxylic acids.

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List of Abbreviations

6-APA	6-aminopenicillanic acid
7-ADCA	7-aminodeacetoxycephalosporanic acid
BSA	Bovine serum albumin
COD	Total Organic Carbon
CSTR	Continuous Stirred Tank Reactor
DMS	Dimethyl sulfide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDAC	Ethylenediaminetetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
FBR	Fluidized Bed Reactor
FTPE	Polytetrafluoroethylene
IPTG	Isopropyl-beta-D-thiogalactopyranoside
OD_{600nm}	Optical density measured at 600nm
PAGE	Polyacrylamide gel electrophoresis
PBR	Packed Bed Reactor
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PLA	Polylactic acid
PMSF	Phenylmethanesulfonyl fluoride
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylene diamine

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Chapter 1

Literature Review

This thesis describes the studies of the biochemical properties of a newly isolated thermostable recombinant amidase from *Bacillus* sp. RAPc8, and the design and development of a continuous reactor process for the production of a target product using an amidase biocatalyst.

1.1. INTRODUCTION

Plants, animals and microorganisms perform a wide variety of non-peptide carbon-nitrogen hydrolysis reactions catalyzed by members of the nitrilase superfamily of enzymes. The enzymes are utilized for nitrile and/or amide hydrolysis reactions, as well as other reactions [Pace and Bremner, 2001] such as oxidation and reduction [Banerjee *et al.*, 2002]. The hydrolysis reactions catalyzed by the nitrilase superfamily of enzymes produce natural products such as auxin, biotin and β -alanine. The reaction mechanism is reported to involve attack of a cyano or carbonyl carbon by a conserved cysteine residue, in the active site of the enzymes [Stevenson *et al.*, 1990, Bork *et al.*, 1994]. The production of a number of valuable natural and synthetic products (e.g. amino acids, amides and carboxylic acids) can be achieved through biocatalysis using these enzymes. Biocatalysis, the application of biological processes in manufacturing and service industries to produce valuable products, has shown enormous potential. The use of enzymes for new and existing applications has rapidly increased in the last two decades. The use of enzymes rather than conventional chemical methods has advantages such as relatively mild conditions of temperature, pH and pressure, specificity of the catalytic activity, and the potential for immobilization and re-use of the biocatalyst.

Biotransformation processes utilize biological catalysts in the form of whole cells or isolated enzymes to catalyze chemical reactions. These processes can be applied in the bioremediation of polluted environments, and the production of active pharmaceutical compounds and fine chemicals. Biotransformation systems are well recognized for their application in chemo-, regio- and enantio- selective reactions where conventional chemical methods would not be adequate, especially for compounds that are natural products [Bull *et al.*, 1998].

1.2. NITRILES

Nitriles are widely spread in nature. In plants, they are mainly present as cyanoglycosides [Banerjee *et al.*, 2002]. Organic cyanides (nitrile-related compounds) defined by the general formula $R-C\equiv N$, exist throughout the natural environment and as intermediates in microbial metabolism [Kobayashi *et al.*, 1989, Cowan *et al.*, 1998], and their synthesis and metabolism by members of the plant, animal and bacterial kingdoms has been well documented [Legras *et al.*, 1990].

Many organisms rely on the toxicity of organocyanide compounds as a defense mechanism against herbivores, predators and parasites. The largest groups of toxic nitrile-related compounds, the cyanogenic glycosides, are mainly produced by plants, often in seeds, roots and the epidermal layers of leaves [Cameron, 2001, PhD thesis]. Plants produce a wide variety of other nitrile compounds such as cyanolipids, ricinine and phenylacetone nitrile, while chemical industries utilize various nitriles for the manufacture of a variety of polymers and other chemicals [Banerjee *et al.*, 2002].

Industry produces nitriles in the form of herbicides, pharmaceuticals and amino acids [Wyatt and Linton, 1988]. Nitriles are mainly utilized in industrial processes either as feedstocks, solvents, extractants, pharmaceuticals, drug intermediates, or pesticides such as dichlobenil, bromoxynil, ioxynil and butiril. Acrylonitrile and adiponitrile, which are starting material for the production of polyacrylonitrile and nylon-6,6-polymers respectively, are typical industrial feedstocks. Nitriles are also used and produced as intermediates in the organic synthesis of amines, amides, amidines, carboxylic acids, esters, aldehydes, ketone and other heterocyclic compounds [Banerjee *et al.*, 2002]. The disadvantage with regard to the industrial application of nitriles is that most of them are highly toxic, mutagenic and carcinogenic in nature [Pollak *et al.*, 1991]. The administration of high levels of nitrile compounds in humans results in gastric problems, nausea, bronchial irritation, respiratory distress, convulsions, coma and osteolthrysm; which may lead to lameness and skeletal deformities [Banerjee *et al.*, 2002]. Therefore, although nitriles are of industrial benefit, their disposal poses a serious health and environmental problem.

Methods for the removal of toxic nitriles from the environment have been developed and are utilized in industry. Microbial degradation has been considered an efficient way of removing these highly toxic nitriles from the environment, and the efficiency of biological methods has been based on their eco-friendly nature [Banerjee *et al.*, 2002]. Microbial degradation is possible because in nature, some microbes contain genes encoding enzymes that are specifically responsible for nitrile metabolism which results in detoxification.

The mechanisms of nitrile metabolism involve hydrolysis (nitrile hydratase, amidase, and nitrilase), oxidation (oxygenase) and reduction (nitrogenase). Hydrolysis is the most common pathway for the microbial metabolism of nitriles [Banerjee *et al.*, 2002]. The hydrolytic conversion of nitriles may occur via one of two established pathways. The nitrile may be hydrolyzed in two steps via an amide intermediate to an acid and ammonia, involving both a nitrile hydratase and an amidase, or it may be hydrolyzed directly to an acid and ammonia via a nitrilase-catalyzed reaction [Figure 1.1].

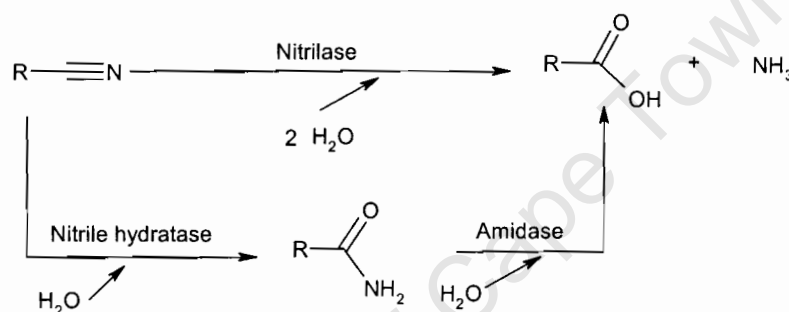


Figure 1.1: Pathways for enzymatic nitrile hydrolysis.

1.3. NITRILE-DEGRADING ENZYMES

Although nitrile-degrading enzymes have been employed in large-scale processes (production of products such as acrylamide, nicotinamide and synthetic antibiotics), they are found relatively infrequently in nature. To date, nitrile-degrading activity has been reported in 3 out of 21 plant families and in a limited number of fungal genera [Banerjee *et al.*, 2002]. Nitrile-degrading activity is more frequently found in the bacterial kingdom, in which nitrile hydrolysis is performed by members of the nitrilase superfamily of enzymes; which have been reported to be nitrilases, aliphatic amidases, β -ureidopropionases, β -alanine synthases, *N*-carbamyl-D-amino

acid amidohydrolases and others. Members of the nitrilase superfamily are classified into 13 branches, in which the substrate specificity of members of 9 of these branches is well known [Pace and Bremner, 2001]. Of the members of 9 these branches whose substrate specificity is well documented (nitriles, acid amides, secondary amides and *N*-carbamyl amides), only one branch is known to have nitrilase activity, whereas 8 branches have apparent amidase or amide-condensation activities [Pace and Brenner, 2001].

Despite the fact that most branches of the nitrilase superfamily are actually amidases, there are many amidases (triad hydrolases, amidase signature enzymes and thiol proteases) that are not related to the nitrilase superfamily. Nitrilases, catalyze the hydrolysis of organic nitriles to their corresponding acids and ammonia, while nitrile hydratases (Nhase), non-members of the nitrilase superfamily, catalyze the hydrolysis of nitriles to their corresponding amides. These are subsequently hydrolyzed to the acids and ammonia by another member of the superfamily, namely amidases. Also, many of the plants, animals and fungi that encode the nitrilase superfamily genes have multiple nitrilase-related proteins from more than one branch of the superfamily [Banerjee *et al.*, 2002].

The physiological role of nitrile-degrading enzymes *in vivo* is not well understood, although in some plants such activities are implicated in nutrient metabolism, particularly in the degradation of glucosinolates [Bestwick *et al.*, 1993] and in the synthesis of the plant growth hormone 3-indole-acetic acid [Bartel and Fink, 1994]. In some higher plants, nitrile-degrading activity is required for cyanide detoxification, while others have also suggested that nitrile-degrading enzymes form part of metabolic pathways that control the production and degradation of cyanogenic glycosides and related compounds [Banerjee *et al.*, 2002].

Other than the nitrilase superfamily of enzymes, other microbial enzymes are also responsible for the metabolism of nitriles such as hydrogen cyanide, which is abiotically formed from various metal cyanides, to produce various products [Banerjee *et al.*, 2002]. Oxygenases, produced by many plants and insects, oxidize various nitriles to cyanohydrins which are further converted to their corresponding aldehydes and hydrogen cyanide by oxynitrilases [Johnson *et al.*, 2000]. In the microbial kingdom, this type of enzymatic activity is almost unknown, although the fungus

Trichoderma sp. has been reported to degrade diaminomalenonitrile to produce hydrogen cyanide [Banerjee *et al.*, 2002]. Figure 1.2 shows different pathways of nitrile metabolism involving members of nitrile-degrading enzymes.

In industry, the most widely used method of nitrile conversion is biocatalytic hydrolysis for the production of various valuable products, in which nitrile-degrading activities from *Rhodococcus* N-774, *Pseudomonas chlororaphis* B23 and *R. rhodochrous* J1 have been used in the production of acrylamide and the synthesis of nicotinamide and nicotinic acid from 3-cyanopyridine [Mathew *et al.*, 1988].

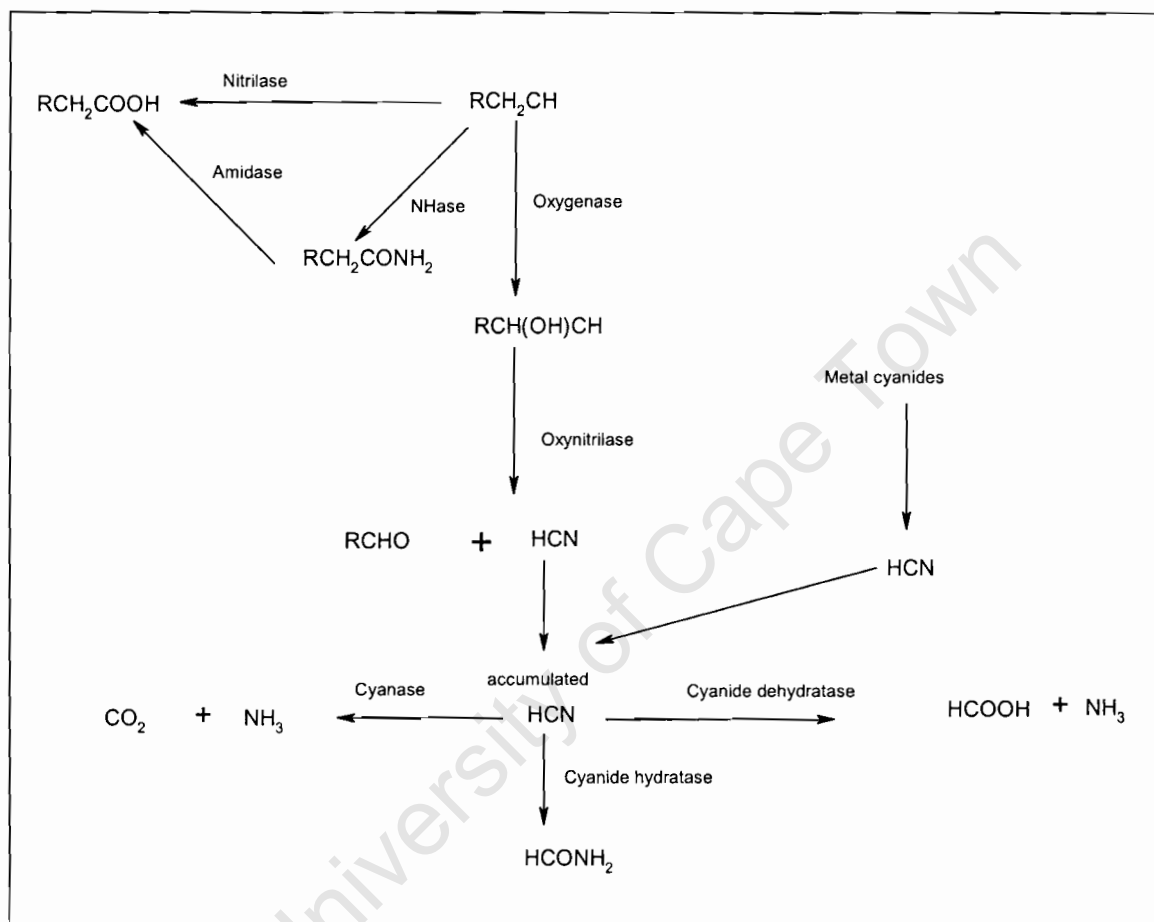


Figure 1.2: An illustration of different pathways of nitrile metabolism [Redrawn from Banerjee *et al.*, 2002] (NHase = Nitrile hydratase).

1.3.1. Nitrilases

Nitrilase, the first nitrile-degrading enzyme to be discovered over 40 years ago, belongs to branch 1 of the nitrilase superfamily of enzymes. Their *in vivo* role in plants is in the production of the plant growth hormone 3-indole acetic acid from 3-acetonitrile [Banerjee *et al.*, 2002]. Currently, a number of enzymes possessing nitrilase activity have been isolated and they have the capability to hydrolyze a wide range of natural and synthetic nitriles. Microbial nitrilases are classified into 3 different categories based on their substrate specificities. The first group includes nitrilases capable of hydrolyzing aromatic or heterocyclic nitriles to the corresponding acids and ammonia. The second group is known to hydrolyze either aliphatic nitriles or arylacetonitriles, while the third group is capable of hydrolyzing both aromatic and aliphatic nitriles [Banerjee *et al.*, 2002]. Nitrilases, in general, do not show the presence of any metal co-factor or prosthetic group in their active site, but they have been reported to use cysteine residues for their catalytic mechanism [Kobayashi *et al.*, 1992a].

1.3.2. Nitrile hydratases

Nitrile hydratases, first discovered in the early 1980s, catalyze the hydrolysis of nitriles to the corresponding amides [Asano *et al.*, 1980]. A number of microorganisms possessing nitrile hydratase activity have been isolated, and their enzymes purified and characterized. Nitrile hydratases are metallo-enzymes containing either cobalt or iron in their active site. Based on the type of a metal co-factor present in the active site of nitrile hydratases, these enzymes are classified into two groups, namely ferric and cobalt nitrile hydratases. Cobalt nitrile hydratases have threonine in a –Val-Cys-(Thr/Ser)-Leu-Cys-Ser-Cys amino acid sequence in the active site, whereas ferric nitrile hydratases have serine. Furthermore, the difference in the metal co-factors may be attributed to the different amino acid residues present in the active site [Banerjee *et al.*, 2002]. Finally, in place of a tryptophan residue, which may be involved in substrate binding, in the cobalt-containing enzymes, ferric-containing enzymes have a tyrosine residue. This is believed to be responsible for the preference of cobalt nitrile hydratases for aromatic substrates rather than for aliphatic nitriles.

1.3.3. Amidases

Amidases catalyze the hydrolysis of carboxylic acid amides (which are nitrile intermediates) to free carboxylic acids and ammonia. Amidases belong to branches 2 and 3 of the nitrilase superfamily of enzymes and they are involved in nitrogen metabolism in cells. They have been found in prokaryotic and eukaryotic cells, as for example in bacteria related to the group of *Nocardia*-like actinomycetes (*Nocardia*, *Rhodococci*, *Arthrobacter*) [Asano *et al.*, 1982, Kobayashi *et al.*, 1993, Mayaux *et al.*, 1990, Maestracci *et al.*, 1988]. To date, amidases have been most extensively characterized in the bacterial kingdom. The substrate specificity and biological functions of these enzymes vary widely, but they generally involve carbon/nitrogen metabolism in prokaryotes, through amide hydrolysis [Banerjee *et al.*, 2002].

Some amidases can only catalyze the hydrolysis of aliphatic amides [Asano *et al.*, 1982, Kobayashi *et al.*, 1993, Mayaux *et al.*, 1990, Maestracci *et al.*, 1988, Thiery *et al.*, 1986], others hydrolyze amides of aromatic acids [Mayaux *et al.*, 1990, Maestracci *et al.*, 1988, Thiery *et al.*, 1986, Hirrlinger *et al.*, 1996], and still others hydrolyze amides of *alpha*- or *omega*-amino acids. Some amidases are highly stereo-specific and others less so. Up till 1996, an amidase associated with a metal co-factor such as cobalt or iron in its active site had only been reported in the case of *K. pneumoniae*, unlike nitrile hydratases [Nawaz *et al.*, 1996].

A number of studies of amidase classification [Chebrou *et al.*, 1996, Fournand and Arnaud, 2001] have showed that bacterial aliphatic amidases fall into two categories. The first group, the nitrilase-related family, includes aliphatic amidases which hydrolyze only short-chain aliphatic amides. These enzymes are typically homohexamers of approximately 230kDa, and contain a cysteine residue which is believed to act as the catalytic nucleophile. This group of enzymes is also known as the wide spectrum amidases and they do not contain the central conserved GGSS-signature in their amino acid sequence which is common to all other enzymes in the amidase family.

The second group includes aliphatic amidases which hydrolyze mid-length amides, some arylamides, α -aminoamides and α -hydroxyamides. These enzymes often demonstrate enantioselectivity, and they belong to the group of the GGSS-signature-containing amidase family [Chebrou *et al.*, 1996]. Kobayashi *et al.* (1997) have reported that these enzymes contain asparagine and serine residues in their active site in place of the most common cysteine residue. With the exception of *Bacillus sp.* BR449 amidase, all nitrile hydratase coupled amidases belong to this class, and are typically homodimers with a native molecular weight of approximately 110kDa [Cameron, 2001, PhD thesis].

The proposed reaction mechanism for amidases is shown in Figure 1.3 [Banerjee *et al.*, 2002]. The carbonyl group of the amide undergoes nucleophilic attack, resulting in the formation of a tetrahedral intermediate which is converted to an acyl-enzyme with the removal of ammonia, and subsequently hydrolyzed to the acid. Amidases also exhibit an acyl transfer activity leading to the formation of hydroxamic acids in the presence of an acyl donor such as hydroxylamine [Fournand & Arnaud, 2001].

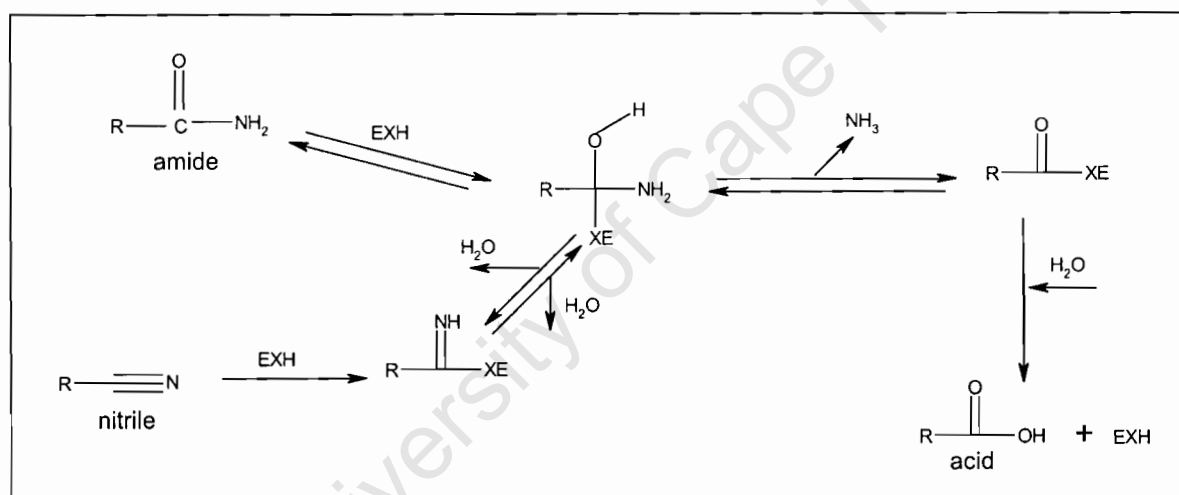


Figure 1.3: The mechanism of the amidase-catalyzed reaction [redrawn from Banerjee *et al.*, 2002].

1.4. THERMOSTABLE AMIDASES

The stability of a biocatalyst is one of the most important factors in determining the productivity in a biocatalytic process. Generally, thermophilic bacteria produce thermostable enzymes. Some of the advantages of using thermostable enzymes are that 1) The enzymes are stable at higher temperatures in which other contaminating microorganisms are unable to grow; 2) Higher reaction rates and lower diffusion limitations can be achieved at higher temperatures; 3) Solubility of substrates and products may be higher, and 4) Favorable equilibrium conditions may be achieved in endothermic reactions. Furthermore, many thermostable proteins have been described with a high level of resistance to degradation by proteolysis and to denaturation by organic solvents, detergents and chaotropic agents such as urea and guanidine hydrochloride [Daniel *et al.*, 1982, Cowan, 1995].

Very few novel thermostable amidases have been reported in literature to date. Those reported include the thermostable nitrile hydratase/amidase from the thermophilic strains *Bacillus pallidus*, *Bacillus smithii* *Bacillus* sp., and *Pseudonocardia thermophila*. Another thermostable nitrile hydratase/amidase was isolated from a *Rhodococcus rhodochrous* strain, and was found to operate at higher temperatures than most of the nitrile hydratases from other mesophilic strains [Mylerova and Martinkova, 2003]. Table 1.1 summarizes the few strains reported to produce thermostable nitrile-converting enzymes (i.e. nitrilases, nitrile hydratases and amidases). All of the thermostable enzymes have optimum activities between 50 and 60°C, and pH optima between 6.0 and 9.0 [Graham *et al.*, 2000]. Ciskanik *et al.* (1995) purified and characterized a thermostable enantioselective amidase from *Pseudomonas chlororaphis* B23. Egorova *et al.* (2004) also purified and characterized a thermostable enantioselective amidase from the thermophilic actinomycete *Pseudonocardia thermophila*. The first archaeal amidase to be characterized was isolated from the hyperthermophilic archaeon *Sulfolobus solfataricus*, cloned and over-expressed into *E. coli* [d'Abusco *et al.*, 2001]. Most thermostable amidases are active within a broad range of temperatures, 40 - 80°C, and this property makes them useful as industrial biocatalysts.

Table 1.1: Summary of the few microorganisms reported to encode thermostable nitrile-degrading enzymes.

Microorganism	Enzyme activity	Substrates	Reference
<i>Acidovorax facilis</i> ATCC 55746	Nitrilase	alkylnitriles, benzonitrile	[Gavagan <i>et al.</i> , 1999]
<i>Bacillus</i> sp. BR449	Nitrile hydratase/amidase	acrylonitrile	[Kim and Oriel, 2000]
<i>Bacillus</i> sp. RAPc8	Nitrile hydratase/amidase	acrylonitrile, (cyclo)alkylnitriles	[Graham <i>et al.</i> , 2000]
<i>Bacillus pallidus</i> Dac521	Nitrilase & nitrile hydratase/amidase	alkylnitriles, arylnitriles, heterocyclic nitriles	[Cramp and Cowan, 1999, Almatawah <i>et al.</i> , 1999]
<i>Bacillus smithii</i> SC- J05-1	Nitrile hydratase/amidase	alkylnitriles	[Takashima <i>et al.</i> , 1998]
<i>Pseudonocardia thermophila</i> JCM3095	Nitrile hydratase/amidase	acrylonitrile	[Yamaki <i>et al.</i> , 1997]

1.5. IMMOBILIZATION TECHNIQUES AND IMMOBILIZED AMIDASES

Methods for stabilization of biocatalysts in order to generate reusable and easy-to-handle enzymes have been developed, applied and improved over the years. Reports have suggested entrapment in hydrogels such as calcium alginate, barium alginate, pectate, *k*-carrageenan, polyvinyl alcohol and acrylamide as good immobilization techniques for nitrile-degrading microorganisms. Some of these immobilization techniques have rendered improved thermal or biochemical stability of the biocatalyst [Mylerova and Martinkova, 2003]. However, immobilization can sometimes damage the biocatalyst or even hinder enzyme activity.

1.5.1. Immobilization by adsorption

Immobilization by adsorption is the simplest method of immobilization and involves surface interactions between enzyme and material support (carrier) [Messing, 1976, Woodward, 1985]. Interactions are due to very weak electrostatic forces such as van der Waals forces, ionic and hydrogen bonding. The immobilization procedure involves mixing of the enzyme and a support material under suitable conditions for a period of time, followed by collection of the immobilized material and extensive washing to remove unbound enzyme [Bickerstaff, 1997]. Commonly used support materials for adsorption are inorganic material including silica, alumina, porous glass and ceramics; organic material including cellulose, starch and activated carbon; and ion-exchange resins including amberlite, sephadex and dowex. The main advantages of this type of immobilization technique are that it is simple, cheap and quick. The support undergoes no chemical change; the method offers little or no damage to the enzyme and is reversible to allow regeneration with fresh enzymes [Bickerstaff, 1997]. The main disadvantages of this type of immobilization technique are enzyme leakage, nonspecific binding and steric hindrance by the support.

1.5.2. Immobilization by cross-linking

Cross-linking is support-free and involves linking enzyme molecules together to form large, three-dimensional complex aggregates. This can be achieved by either physical or chemical methods [Broun, 1976]. Flocculation of cells is a well-known physical cross-linking method in the biotechnology industry. Flocculating agents including polyamines, polyethyleneimine, polystyrene sulfonates, and various phosphates have been used extensively and are well characterized [Bickerstaff, 1997]. Chemical cross-linking methods involve covalent bonding of enzyme molecules using a bi- or multifunctional reagent such as glutaraldehyde or toluene diisocyanate. The main disadvantage of this type of method is the toxicity of most cross-linking reagents. Furthermore, this method is rarely used as the sole means of immobilization because of poor stability and lack of mechanical properties [Bickerstaff, 1997]. Therefore cross-linking is most often used to enhance other immobilization methods.

1.5.3. Immobilization by entrapment

Immobilization by entrapment, unlike adsorption and covalent binding, allows enzyme molecules to be in solution, but restricted in movement by the lattice structure of a gel/matrix [Bickerstaff, 1995, O'Driscoll, 1976]. Entrapment can be achieved by mixing enzyme molecules with a poly-ionic polymer material and then cross-linking the polymer with multivalent cations in an ion-exchange reaction to form a lattice structure that traps the enzyme. Alternatively, enzyme molecules can be mixed with chemical monomers that are then polymerized to form a cross-linked polymeric network, trapping the enzyme inside the lattice [Bickerstaff, 1997]. For the latter method, a number of acrylic monomers are available for the formation of hydrophilic copolymers. For example, acrylamide monomers are polymerized to form polyacrylamide, while methylacrylates are polymerized to form polymethacrylate.

1.5.4. Immobilization by covalent Binding

This method involves covalent bonding between enzyme molecules and a support material. The bond is normally formed between functional groups present on the surface of the support and amino acid residues on the surface of the enzyme molecule. The amino acid residues most often involved in covalent bonding are the amino group ($-NH_2$) of lysine or arginine, the carboxylic group ($-CO_2H$) of aspartic acid or glutamic acid, the hydroxyl group ($-OH$) of serine or threonine, and the sulfhydryl group ($-SH$) of cysteine [Srere and Uyeda, 1976]. A variety of support materials are available for covalent binding and therefore the choice of a support material depends on its physical and chemical properties. Research has shown that hydrophilicity of the support material is the most important factor for maintaining enzyme activity in a support material [Gemeiner, 1992]. Hydrophilic polysaccharide polymers including cellulose, dextran, starch and agarose are more often used for enzyme immobilization, and other supports include porous glass, Eupergit beads and porous silica.

1.5.5. Immobilized amidases

In industry, amidase activity is mostly used as immobilized whole cells, including whole cells of the third generation *Rhodococcus* J1, *Rhodococcus* N-774 and *Pseudomonas chlororaphis* B23 and *R. rhodochrous* J1 strain used for the production of acrylamide, nicotinamide and nicotinic acid [Mathew *et al.*, 1988]. Studies have shown that improved biochemical properties of enzymes can be achieved through immobilization. For example, whole cells of *Bacillus pallidus* immobilized by entrapment in calcium alginate were more resistant to high concentrations of 3-cyanopyridine and nicotinic acid than free cells [Almatawah and Cowan, 1999], and whole cells of *Candida guilliermondi* immobilized in alginate beads were capable of hydrolyzing substrates that were not hydrolyzed by free cells of the same microorganism [Dias *et al.*, 2001].

Purified isolated amidases, including the most widely used amidase industrially, the penicillin G amidase, which is used for the production of semi-synthetic antibiotics, have been immobilized successfully [Böck *et al.*, 1983]. Other isolated amidases which have also been immobilized successfully includes the penicillin acylase from *Kluyvera citrophila* which was immobilized on glutaraldehyde derivatives of silanized controlled-pore ceramics [Bodhe and Sivaraman, 1987], a penicillin G acylase immobilized on micro-porous silica for the production of 6-aminopenicillanic acid (6-APA) [Cardias *et al.*, 1999], and a thermostable L-aminoacylase from *Thermococcus litoralis* immobilized by entrapment in a polyacrylamide gel [Toogood *et al.*, 2002].

1.6. INDUSTRIAL APPLICATIONS OF AMIDASES

Biotransformation of nitriles has provided great potential for the synthesis of various chemical products. The ability of an enzyme system to convert a cyano- functional group to either an acid or an amide is of great value [Banerjee *et al.*, 2002]. Traditional chemical processes for the conversion of nitriles to their corresponding acids or amides have several disadvantages, including requirement for highly acidic or basic reaction conditions, high reaction temperature, formation of by-products such as HCN, and the generation of large amounts of salt waste, which are of environmental concern. Biotransformation of nitriles allows the use of ambient reaction

conditions and is used industrially, for the chemo-, regio-, or enantio-selective hydrolysis of various nitriles [Fournand and Arnaud, 2001, Sugai *et al.*, 1997].

Several organic compounds, such as *p*-aminobenzoic acid, acrylic acid, nicotinic acid, pyrazinoic acid and 3-indole acetic acid, are commercially produced through the biotransformation of nitriles using amidase activity, in microbial cells [Banerjee *et al.*, 2002]. About 30 000 tons/year of acrylamide are produced by a third generation *Rhodococcus rhodochrous* J1 strain [Yamada and Nagasawa, 1994]. *R. rhodochrous* J1 strain is also used industrially for the production of nicotinamide and nicotinic acid through the conversion of 3-cyanopyridine [Mathew *et al.*, 1988]. 5-cyanovaleric acid, an intermediate in the synthesis of nylon-6 and nylon-6,6-polymers, is produced by a *R. rhodochrous* K22 strain through the conversion of adiponitrile. An *Acremonium* sp. is used for the selective mono-hydrolysis of trans-1,4-dicyano cyclohexane to form a homeostatic drug, tranexamic acid [Banerjee *et al.*, 2002].

Purified immobilized amidases are also utilized industrially for the production of various compounds; in fact, amidases are among the most widely used amide-hydrolyzing enzymes in industry, in their free or immobilized form. The chemo- and regio-selectivities of amidases are utilized in the production of antibiotics (penicillin acylase), the hydrolysis of C-terminal amide groups in peptides (peptide amidase), and the transformation of cyclic imides (half-amidase, imidase) to mention a few [Barsomian *et al.*, 1990, Hoople, 1998, Ogawa *et al.*, 2000, Soong *et al.*, 2000, Stelkes-Ritter *et al.*, 1997, Sugai *et al.*, 1997]. Enantioselective amidases are used in the production of optical active D- or L- α -amino acids, hydroxycarboxylic acids, or α -methylarylacetic and α -methoxyarylacetic acids. Figure 1.4 illustrates two reactions utilized for the commercial production of 6-APA and L-pipecolic acid using amidases.

Penicillin G amidase is one of the most widely used amidase superfamily enzymes, at industrial scale. It catalyzes the hydrolysis of penicillin G to phenylacetic acid and 6-aminopenicillanic acid (6-APA), which is a critical starting compound for the synthesis of many novel β -lactam antibiotics [Figure 1.4] [Böck *et al.*, 1983]. Another class of amidases, the penicillin acylases, are also used in the industrial production of semi-synthetic penicillins and cephalosporins, which remain the most widely used group of antibiotics [De Vroom, 1999]. The industrial method used

for the production of 6-aminopenicillanic acid has undergone change due to the fact that enzyme-catalyzed processes have replaced traditional chemical synthesis [Arroyo *et al.*, 2003]. Some years ago 6-APA and 7-ADCA were produced chemically, based on the splitting of penicillin G and cephalosporin G, but this chemical method was subject to complicated technical requirements and the use of toxic chemicals causing environmental pollution, as well as impurities in the products.

D-amino acids are important chiral intermediates for pharmaceuticals, agrochemicals and food additives. The production of D-amino acids can be achieved by the resolution of chiral racemates [Wakayama *et al.*, 2003]. At present, enzymatic chiral resolutions have been reported for lipase, protease, aminoacylase, amidase and hydantoinase. An *L*-aminoacylase, a representative member of the amidase superfamily, was the first to be used industrially [Bradbury *et al.*, 1982].

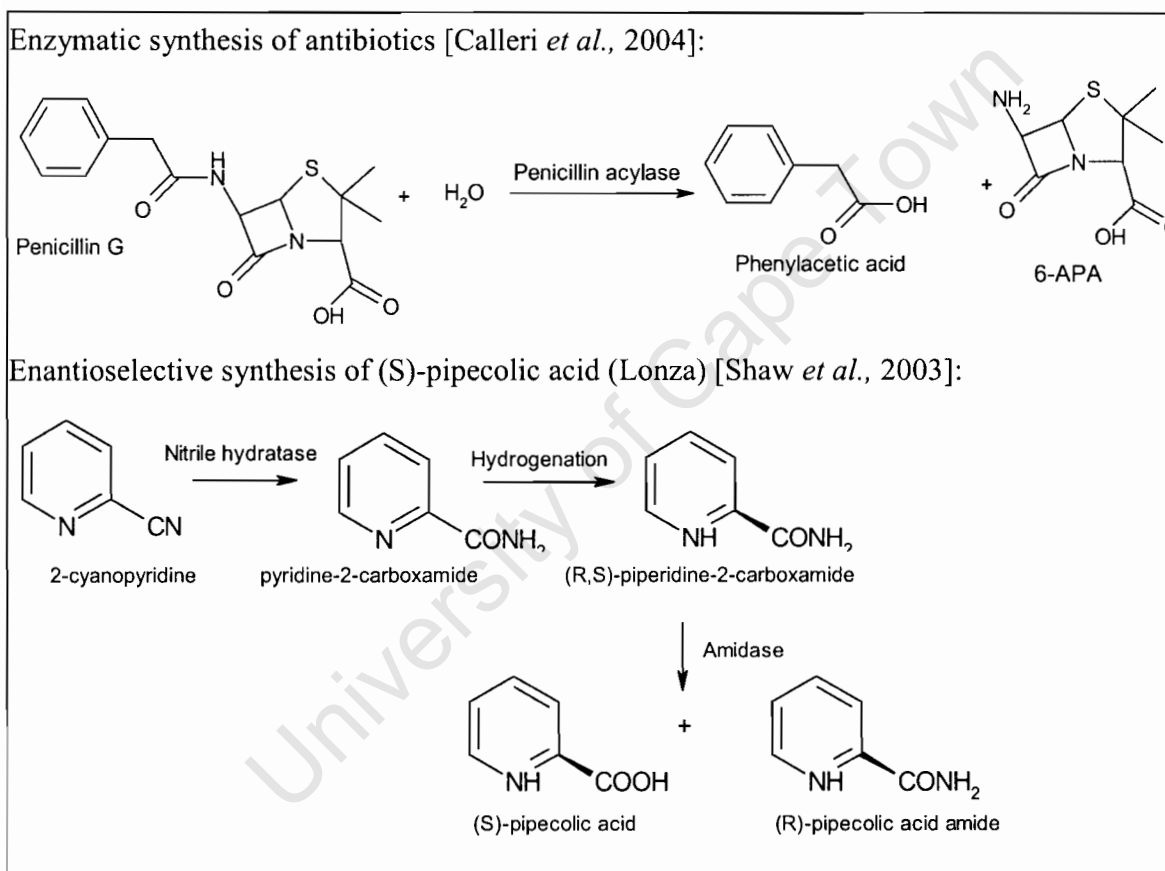


Figure 1.4: An illustration of amidase catalyzed industrial reactions.

1.7. OTHER NOVEL APPLICATIONS OF AMIDASES

Currently, traditional chemical and fermentation processes are the main methods used for the commercial production of carboxylic acids. As discussed earlier, amidases are used industrially for the production of a few acids such as nicotinic acid, *p*-aminobenzoic acid, pyrazinoic acid, 3-indole acetic acid, (*S*)-pipecolic acid and 6-APA. Research on amidases has shown that these enzymes can also be used for the production many other products, which can potentially be scaled-up to industrial level. Some laboratory scale studies have showed that the anti-inflammatory drug, *L*-ibuprofen, can be produced with an enantiomeric excess of 95%, using whole cells of *Acinetobacter* sp. AK226 [Yamamoto *et al.*, 1990]. In bioremediation, the effluent from acrylonitrile manufacturing industries was degraded to about 75% reduction in COD and 99% removal of detectable toxic compounds, using mixed cultures of bacteria, containing nitrile-hydrolyzing enzymes, in batch and continuous cultures [Wyatt and Knowles, 1995]. Further research could lead to the commercialization of these and similar processes, thereby offering new methods as alternatives to the current commercial methods.

1.8. INDUSTRIAL REACTORS FOR AMIDASE APPLICATION

Biocatalytic reactors are engineered systems in which the activity of biocatalysts is utilized for the synthesis of products such as antibiotics, antibodies, proteins, and drugs, and other chemical products. Biocatalytic reactors may differ from traditional reactors because of deviations in their dynamic behavior due to metabolic regulation in cells [Ramkrishna, 2003]. Industrial enzyme reactions are carried out in a variety of reactors which have different operational properties. The choice of a reactor configuration is dependent on, amongst other things, its cost and ease of operation [Sinnott, 1993]. Furthermore, enzyme kinetics, substrate and product inhibition, and the choice of using whole cells or isolated enzymes, may also influence the choice of a reactor-type for a particular biotransformation. Many different types of biocatalytic reactors have been designed and applied in industrial processes, but the most widely used reactor configurations include batch, stirred tanks, packed beds, membrane and fluidized bed reactors.

1.8.1. Stirred Batch reactors

A basic Stirred Batch Reactor consists of a tank fitted with an agitating device. A heating or cooling jacket for temperature control and baffles to ensure adequate mixing are usually fitted to the reactor. In stirred batch reactors, the enzyme is either added to the solution inside the tank or an immobilized enzyme is dispersed in the reaction mixture. [<http://www.food.reading.ac.uk/online/fs560/topic2/t2c/t2c.htm>]. Some of the most significant advantages of stirred tanks are that they are simple to use and very versatile. The main disadvantage is that stirred tanks must be emptied and refilled at the end each batch run, and this leads to loss of productivity and downtime and potential damage to the biocatalyst.

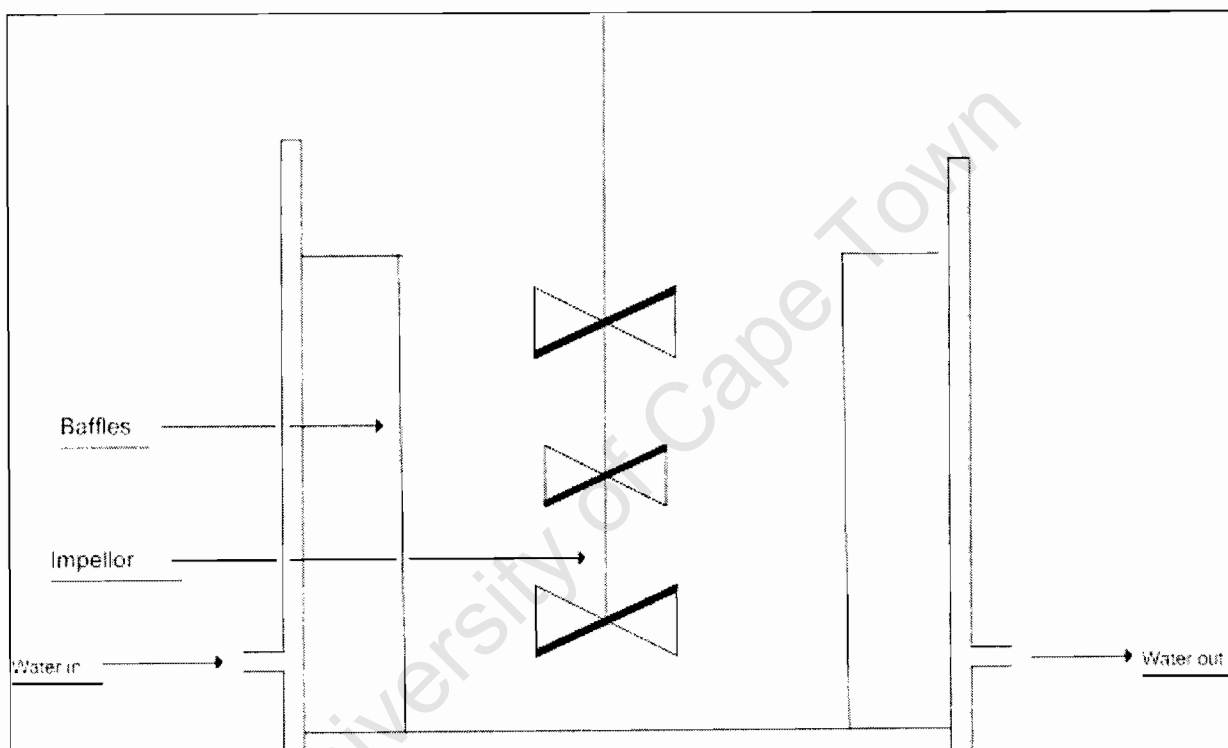


Figure 1.5: An illustration of a stirred batch reactor configuration with a heating/cooling system.

1.8.2. Continuous Stirred Tank reactors

A Continuous Stirred Tank Reactor (CSTR) is basically a stirred tank with a continuous addition and removal of substrate and product, respectively. The enzyme in a CSTR can be attached in the same manner as in stirred batch. The continuous addition of substrate and removal of product is particularly desirable for substrate- or product-inhibited enzymes [<http://www.food.reading.ac.uk/online/fs560/topic2/t2c/t2c.htm>]. In general, most enzymes, including amidases, lose activity at high concentrations of substrate and/or product. Therefore, continuous reactors are more favoured for enzyme-catalyzed reactions. One advantage of CSTRs is the ease of monitoring operating parameters such as temperature, pH and dissolved oxygen. However, low substrate concentration due to the continuous addition can reduce the effectiveness factor of the enzyme and thus lead to inefficient use of the enzyme.

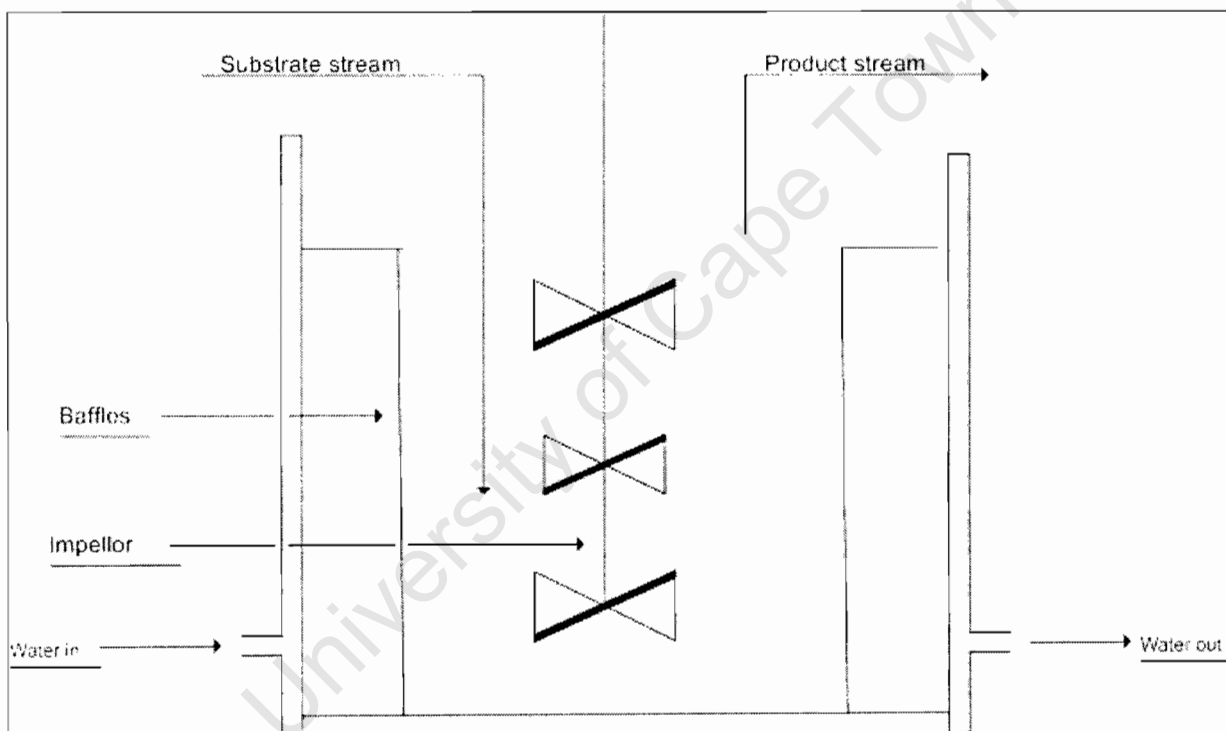


Figure 1.6: An illustration of a Continuous Stirred Tank reactor configuration with a heating/cooling system.

1.8.3. Packed Bed reactors

In a Packed Bed Reactor (PBR), the enzyme must be utilized in its immobilized form. The immobilized-enzyme particles are held fixed inside the column and the substrate is pumped through the column. Depending on the substrate and/or product flow properties (i.e. viscosity), operating costs and other costs, the pumping action of the substrate stream can either be upward or downward [http://www.food.reading.ac.uk/online/fs560/topic2/t2c/t2c.htm]. The main advantage of this type of reactor is that there is no further separation of the product from the enzyme, due to the immobilization. In general, enzymes are immobilized on mechanically strong carriers such as glass beads and Eupergit beads, for application in packed-bed reactors. The main disadvantage of PBRs is the difficulty in maintaining even flow and constant temperature. Also, enzyme particles at the entrance of the reactor are usually exposed to higher substrate concentration whereas the enzyme particles at the exit are exposed to higher product concentration, which can be a disadvantage for substrate- or product-inhibited enzymes [http://www.food.reading.ac.uk/online/fs560/topic2/t2c/t2c.htm].

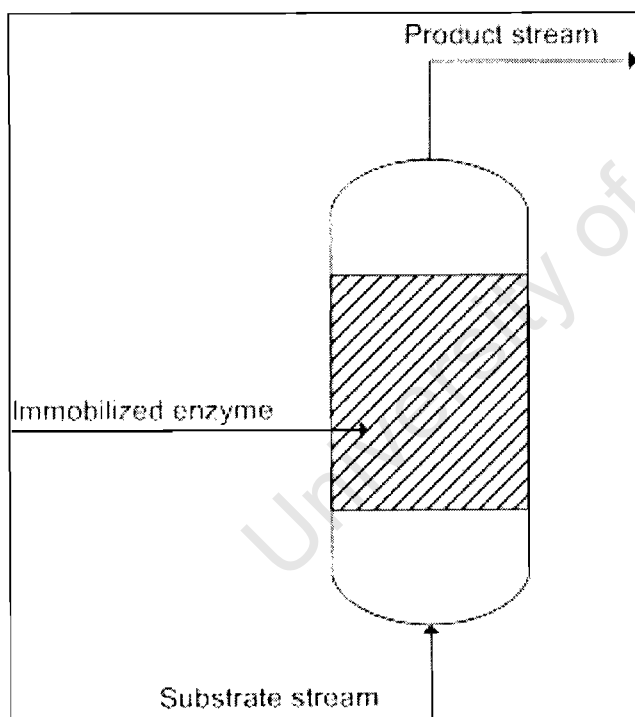


Figure 1.7: An illustration of a fixed/packed bed reactor configuration.

1.8.4. Fluidized Bed reactors

In a Fluidized Bed Reactor (FBR), the enzyme must also be used in its immobilized form. The immobilized enzyme particles are packed in a bed inside the column, where the properties of the immobilization carrier must include floatability and resistant to shear stress. A high rate upward motion of the substrate stream fluidizes the immobilized enzyme bed. The most significant advantage of fluidized bed reactors is the high mass transfer rates, due to high fluidization velocities. Therefore it is rare that a reaction in a fluidized bed reactor will be mass transfer limited. The theoretical disadvantage of fluidized beds is that they are difficult to scale up and therefore their use is generally restricted to small-scale, high value products [<http://www.food.reading.ac.uk/online/fs560/topic2/t2c/t2c.htm>].

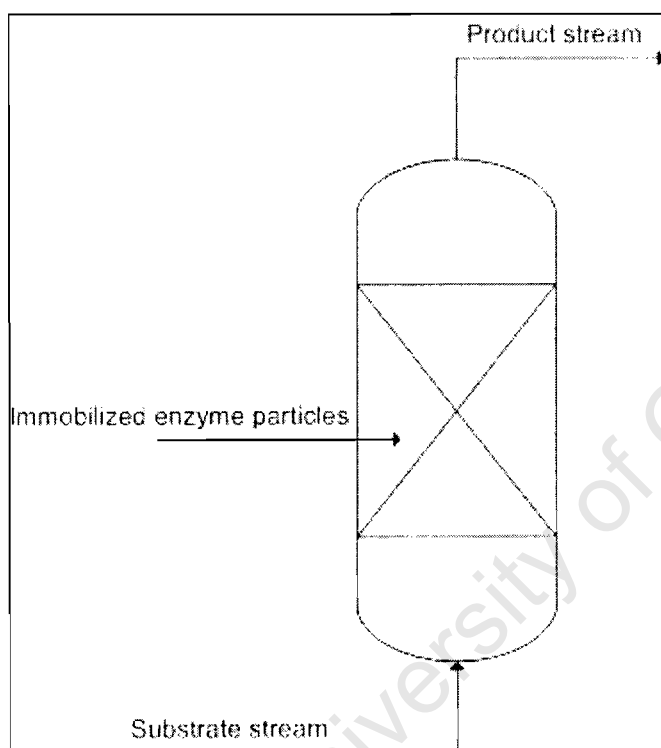


Figure 1.8: An illustration of a fluidized bed reactor configuration.

1.8.5. Membrane reactors

In a membrane reactor, the enzyme is either immobilized on the membrane itself or immobilized on another carrier, which is then packed inside the membrane. Ultimately, the immobilized enzyme must be contained within a semi-permeable membrane immersed in a stirred tank. The tank is then filled with a substrate-buffer mixture and stirred to allow diffusion in and out of the membrane. Membrane reactors can be operated in either batch or continuous mode. The main advantage of membrane reactors is that they are easy to use, but they are difficult to model mathematically. They are often used for small-scale production and applications involving multi-enzyme systems and co-factor recycle. The theoretical disadvantage of membrane reactors is the high cost of membrane fibres [<http://www.food.reading.ac.uk/online/fs560/topic2/t2c/t2c.htm>].

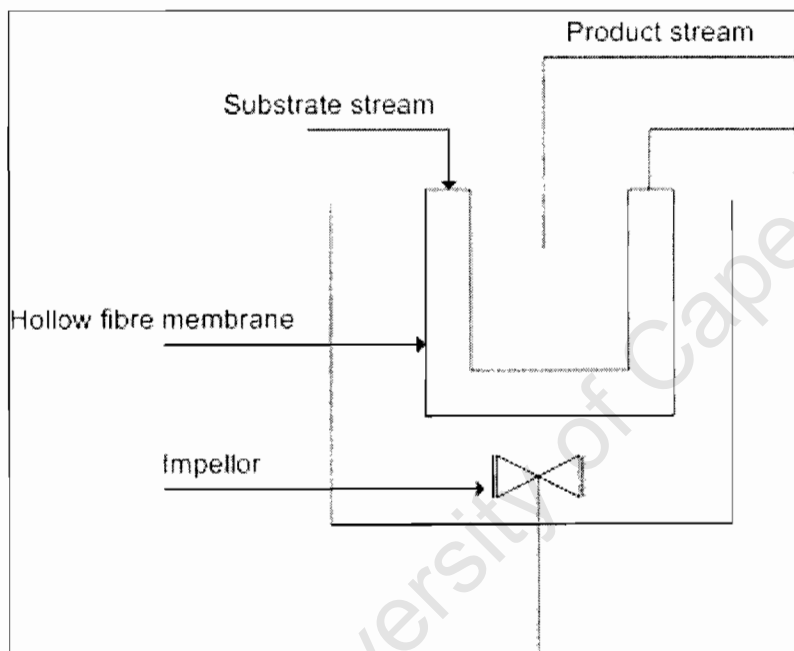


Figure 1.9: An illustration of a membrane reactor configuration.

1.9. CONCLUSION

This literature survey has described the occurrence of nitrile and nitrile-related compounds, some of which occur in the environment. Some nitriles compounds (e.g. acrylonitrile, acetonitrile) are used for the production of commercial products such as nylon polymers, while others (e.g. herbicides) have been reported to pose health and environmental problems due to their high toxicity. This survey has also described the occurrence of microorganisms capable of converting both synthetic and naturally-occurring nitriles to other intermediates and/or products. Therefore, these microorganisms could offer a potential solution for dealing with nitrile compounds, through biotransformation to various valuable products (i.e. amides, acids). Few industrial scale processes have been developed for the conversion of nitriles to useful products, using nitrile-degrading enzymes and yet biotransformation reactions using such enzymes (amidases, nitrile hydratases) have the potential to become commercial processes. These processes have the potential to replace traditional chemical processes due to their eco-friendly nature, and selectivity characteristics. The classification of amidases reveals that these enzymes exhibit broad substrate selectivities, ranging from aliphatic to aromatic amides. Also, some amidases possess enantioselectivity and can be used to produce chiral compounds, while others are capable of operating at high temperatures, at which high reaction rates can be achieved. Based on the success of the few amidases already used industrially and the potential of others, it can be concluded that in the future, more biocatalytic processes will be utilized at industrial scale, in place of traditional chemical methods.

In conclusion, this literature survey shows that the presence of nitrile-related waste in the environment poses huge environment impact, but nitrile-degrading microorganisms capable of converting these nitriles into useful products have been studied and are well characterized. Therefore, these microorganisms could be utilized at industrial scale, either as whole cells or isolated enzymes, for the degradation of nitriles and nitrile-related compounds into useful products such as carboxylic acids, optical pure amino acids and synthetic antibiotics.

1.10. AIMS AND OBJECTIVES

This research is part of a collaborative project in which the primary objective is to screen, isolate and identify nitrile- and amide-metabolizing enzymes from thermophilic microorganisms. The enzymes are then studied for potential application as industrial biocatalysts for the production of various products. In this research, the main objectives were:

- 1) Production and purification of the recombinant amidase isolated from *Bacillus* sp. RAPc8.
- 2) Primary characterization of the free amidase in terms of operating parameters such as temperature, pH and thermostability.
- 3) Determination of the amidase substrate specificity and chiral selectivity.
- 4) Investigation of potential inhibition by co-solvents, sulfhydryl reagents, chelating reagents and protease inhibitors.
- 5) Immobilization of the amidase with different immobilization techniques and selection of a suitable immobilization method.
- 6) Primary characterization of the immobilized enzyme in terms of operating parameters such as temperature, pH and thermostability.
- 7) Selection and production of a target product.
- 8) Development of a continuous process for the production of the target product.
- 9) Application of a mathematical model to the reactor process and comparison with the model to experimental data.

1.11. PROJECT FOCUS

The main focus of this project was to study the biochemical properties of the recombinant amidase from *Bacillus* sp. RAPc8 and to develop a continuous reactor process for the production of a target product. Ultimately, the broader project is focused on developing a potential industrial biocatalyst and demonstrating its ability to catalyze an industrially relevant process.

1.12. STRUCTURE OF THE THESIS

This thesis is divided into 6 main chapters, in which chapter 1 surveyed the literature of nitrile-degrading microorganisms, focused mainly on amidases, their classification and functions. Therefore, in chapter 1, in depth discussion of amidases including their current industrial applications, other novel applications and industrial reactors used for amidase-catalyzed reactions, is presented. Experimental research work is described in chapter 2, whereby the amidase from *Bacillus* sp. RAPc8 was produced by fermentation using a commercial fermentor. In this chapter, the focus was on the production of high quantity (protein concentration) and highly active amidase enzyme through high levels of expression, extraction and purification by heat-treatment. After successful production of the amidase, purified extracts of the enzyme were used to study its biochemical properties including optimal operating conditions, substrate and chiral selectivity, as reported in chapter 3. In the work described in chapter 4, the amidase was immobilized using a number of modern immobilization techniques, and the choice of the best technique was based on the recovery of high amidase activity and protein concentration, after immobilization. Immobilization was carried out in order to develop a potential re-usable biocatalyst for industrial applications. Also in the work reported in chapter 4, the biochemical properties of the immobilized amidase were studied and compared to those of the free amidase, in order to determine whether immobilization has changed the characteristics of the amidase. Based on the biochemical properties of the amidase as discussed in chapters 3 and 4, a target product was selected. This work is described in chapter 5. Batch studies were carried out in order to determine the productivity of the amidase towards the production of the target product, in terms of reaction rates, initial rate kinetics and inhibition studies. Based on the batch studies described in chapter 5, a continuous reactor process was designed and developed, as described in chapter 6. The reactor was then used to demonstrate the potential ability of the amidase biocatalyst to continuously produce the target product on laboratory scale. Mathematical models were used to predict the reactor productivity towards the target product under operating conditions which vary from the experimental conditions. The mathematical models could also be used for scale-up of the reactor. General conclusions drawn from the results described in chapters 2 to 6, were presented in chapter 7, and recommendations were made based anticipated future work.

Chapter 2 Production and purification of the recombinant amidase from *Bacillus* sp. RAPc8 cloned into *E. coli* BL21 strain pNH 223 pLySs

2.1. INTRODUCTION

A number of microorganisms, both recombinant and wild-type, possessing amidase activities, have been successfully grown, and their enzymes purified. These biocatalysts are used, either as whole cells or isolated enzymes, for the biocatalytic hydrolysis of various amides. Amidases are often produced by recombinant microorganisms, such as *E. coli*, cloned with antibiotic resistant genes. The reason for this is that most of the wild-type strains, from which the amidases originate, are difficult to grow in a laboratory or are completely unculturable. Therefore, the production of amidases using recombinant strains is used and high-level expression of the enzyme can be achieved.

A new thermostable amidase was isolated from a *Bacillus* sp. RAPc8 strain, and the gene encoding the amidase was over-expressed into *E. coli* BL21 strain pNH 223 pLySs [Cameron, 2001, PhD thesis]. The work by Cameron (2001) also involved growth, expression and purification of the amidase protein after successful fermentation of the recombinant strain in flask culture [Cameron, 2001, PhD thesis], and some preliminary work on biochemical properties of enzyme in terms of optimum working pH, temperature and thermostability.

In this chapter, the growth curve and expression profile of the recombinant amidase from laboratory scale fermentation are reported. The enzyme was extracted and purified according to the method of Cameron (2001), and the results were compared with other literature reports on production of recombinant amidases.

2.2. MATERIALS AND METHODS

2.2.1. Materials

All chemicals were purchased from Sigma-Aldrich (SA), Aldrich-SA and Fluka. All solvents were purchased from Merck-SA. Fresh colonies of the amidase expression system in plate culture were received from Arcam Laboratory, University of the Western Cape.

2.2.2. Growth and Expression of the amidase gene

This method was adapted from Cameron (2001). Fresh colonies of *E. coli* BL21 strain pNH 223 pLySs were used to inoculate 25ml nutrient broth medium supplemented with 25µl carbenicillin solution (50mg/ml) and grown overnight. The cells were centrifuged at 8000rpm for 10 minutes, 4°C, washed once with nutrient broth to remove secreted β-lactamase, resuspended in nutrient broth and used to inoculate a 250ml nutrient broth flask supplemented with 250µl of 50mg/ml carbenicillin solution. The cultures were grown at 37°C until optical density $A_{600nm} \approx 0.60$ was reached. At this point, expression of the recombinant protein was induced by addition of 0.4mM final concentration of IPTG and incubation was continued for an additional 3-4 hours.

2.2.3. Fermentation of the *E. coli* BL21 pNH 223 pLySs strain

A New Brunswick Scientific BIOFLO 110 series fermentation system, fitted with online pH, dissolved oxygen and temperature measuring devices, was used for the fermentation of the *E. coli* BL21 strain pNH 223 pLySs strain. 500ml of the *E. coli* pNH 223 pLySs culture grown overnight was used to inoculate approximately 4.5L of nutrient broth media supplemented with 4.5ml of 50mg/ml carbenicillin solution. Fermentation conditions were 37°C temperature, 3.0vvm aeration, 500rpm agitation and an initial pH of 6.8. 20ml samples were withdrawn every 30 minutes and amidase induction, using 0.4mM final concentration of IPTG, was initiated when the optical density was approximately 0.60. Fermentation was then continued for a further 4 to 5 hours with samples withdrawn every 60 minutes. The Growth curve and expression profile of the amidase were determined from the samples.

2.2.4. Extraction and Purification of the enzyme

E. coli BL21 pNH 223 pLySs cells were harvested by centrifugation at 8000rpm for 15 minutes at 4°C. Cell pellets were washed with 50mM potassium phosphate buffer (pH 7.0), centrifuged, and resuspended in appropriate amount of 50mM potassium phosphate buffer (pH 7.0). Cell lysis was achieved by freezing the cells at -20°C overnight and thawing at room temperature. Cell disruption was achieved by 6-10 cycles of 2.0 min sonication bursts followed by 30 seconds breaks. The extract was cleared by centrifugation at 10000rpm for 15 minutes at 4°C. Soluble proteins were recovered in the supernatant and stored at -20°C until further use. Amidase purification was achieved by incubating the soluble proteins sealed in 50ml Falcon tube, in a water bath at 75°C for 45 minutes. After incubation and precipitation of superfluous proteins, the sample was centrifuged at 10000rpm for 15 minutes at 4°C. The supernatant was transferred into a fresh 50ml Falcon tube and stored at -20°C until further use.

2.2.5. SDS-PAGE gel electrophoresis

Homogeneity and molecular weight of the amidase were determined using SDS-PAGE denaturing gel electrophoresis. The gel was run with a 30% polyacrylamide in the presence of 0.1% SDS at 50mA and 120V using the method adapted from Laemmli (1970). Denatured samples were prepared by boiling equal amounts (20µl) of protein and SDS sample buffer at 100°C for 5 minutes. The gel was stained with a 0.25% Coomassie Brilliant Blue R-250 solution containing 10% and 50% glacial acetic acid and absolute methanol respectively. Excess stain was removed using a destaining solution of 10% glacial acetic acid.

2.2.6. Protein determination assay

Protein concentration was determined using the Bradford Coomassie Brilliant Blue dye-binding assay [Cameron, 2001, PhD thesis, Bradford, 1976]. Purified enzyme samples (20µl) were added to 1ml of 5x diluted Bradford solution in a cuvette. The protein concentration was determined spectrophotometrically at 595nm after 5 minutes of incubation at room temperature. Protein standards were prepared using bovine serum albumin.

2.2.7. Activity determination assay

Amidase activity was determined based on the release of ammonia from the conversion of amides to their corresponding acids and ammonia using the phenol-hypochlorite ammonia detection method [Cameron, 2001, PhD thesis, Weatherburn, 1967]. Unless otherwise specified, all amidase-catalyzed reactions were performed using this method, in which aliquots of the reaction mixture (100µl) were added into 350µl Reagent A (0.59M Phenol and 1mM Sodium nitroprusside) of the ammonia detection method. This was followed by the addition of 350µl Reagent B (2.0M Sodium hydroxide and 0.11M Sodium hypochlorite) and the activity was measured spectrophotometrically at 600nm after 15 minutes incubation at room temperature. Activity standards were prepared using ammonium chloride.

2.3. RESULTS

2.3.1. Fermentation of the *E. coli* BL21 pNH 223 pLySs strain

The amidase was produced by fermentation of the recombinant *E. coli* strain using a commercial 7.5L BIOFLO 110 series fermentor system, as described in section 2.2.3. The suitable agitation speed and aeration rates were estimated by observation of the growth medium. At very high agitation and aeration rates, foaming occurred and thus both agitation and aeration rates were set to the highest possible values where only a little foaming occurred (500rpm and 3.0vvm respectively). It must be noted that the BIOFLO 110 series fermentor was run with the absence of antifoam, to prevent potential inhibition. For a 10v/v% inoculum, stationary phase ($OD_{600nm} \approx 0.60$) was reached after about 60 minutes of growth. At this point, the amidase expression was induced by IPTG and the fermentation was continued for 4-5 hours, during which more foaming started occurring. In order to reduce the foaming, the agitation rate was again reduced to a point where little foaming occurred (400rpm). Figure 2.1 shows a snapshot of the BIOFLO 110 series fermentation system about 4 hours after inoculation. The pH of the growth media started at an initial pH of about 6.8 and then increased with fermentation time to about pH 8.3 after 5 – 6 hours of fermentation. The biomass yield was measured using wet weight and was estimated to be about 500mg (wet cells) per litre of broth.



Figure 2.1: A snapshot of the BIOFLO 110 series fermentation system after about 4 hour of inoculation.

2.3.2. Growth curve and Expression profiles

A typical growth curve of the *E. coli* BL21 strain pNH 223 pLySs is illustrated with Figure 2.2. The growth curve followed Monod kinetics with a log-phase under 30 minutes and the stationary-phase was reached after approximately 4 hours of fermentation [Figure 2.2]. Growth of the strain started at an initial optical density of 0.2 and the highest biomass yield was obtained at a maximum optical density of approximately 1.5.

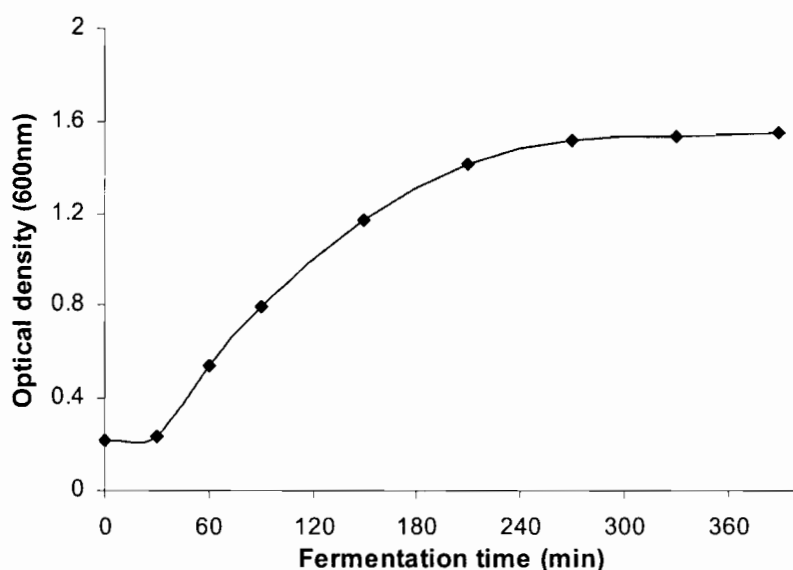


Figure 2.2: Growth curve of the *E. coli* BL21 strain pNH 223 pLySs grown in a BIOFLO 110 series fermentor.

Expression of the amidase gene was induced by the addition of 0.4mM IPTG at the time when $OD_{600nm} \approx 0.60$. High-level expression of the amidase was achieved under the fermentation conditions used, with the specific activity on 25mM acetamide reaching 20U/mg after approximately 4.5 hours of fermentation [Figure 2.3].

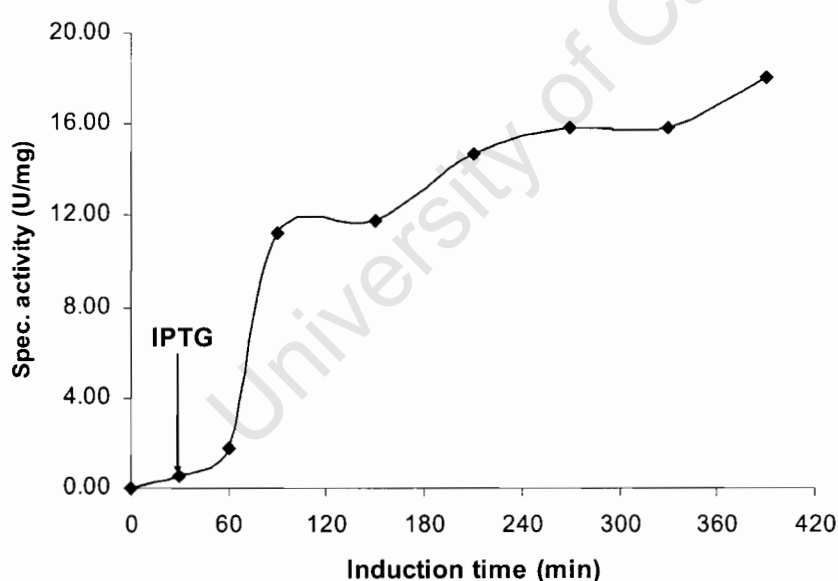


Figure 2.3: Expression curve of the amidase gene after induction using IPTG, in a 5L BIOFLO 110 series fermentor.

2.3.3. Recovery and purification table

The extraction of the amidase protein by sonication and centrifugation resulted in a crude protein extract containing 426mg/ml protein concentration. A single heat-treatment purification of the protein resulted in the precipitation of about 50% (217mg from 426mg containing solution) of unwanted proteins and an increase in total activity of 200% [Table 2.1]. A number of experiments were conducted on the purification of the amidase by heat-treatment, and purification fold and activity yield for all the experiments, ranged between 2.0 – 3.0 and 50 – 70% respectively.

Table 2.1: Purification table for the amidase from *Bacillus* sp.RAPc8, by a single heat-treatment step.

Purification step	Volume (ml)	Total dissolved protein (mg)	Total activity (U/ml)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	50	426	15980	37.5	100	1
Heat-treatment	50	209	30930	148.0	200	2.0

2.3.4. SDS-PAGE gel electrophoresis

Figure 2.4, below, shows that the amidase is a single subunit enzyme with a molecular weight of about 35kDa. The protein-bands shows that the purified extract contained lower amounts of unwanted protein.

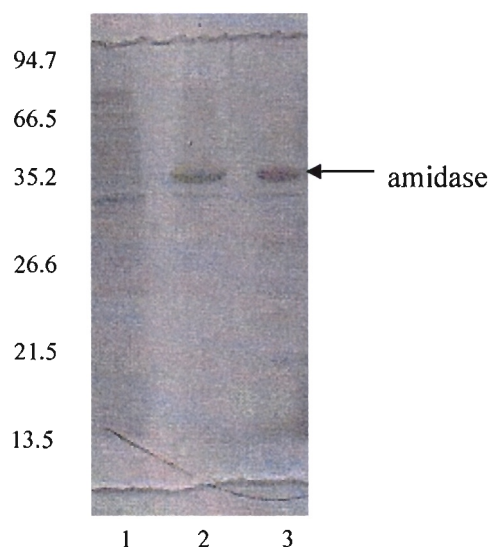


Figure 2.4: SDS-PAGE gel electrophoresis: 1. Uninduced *E. coli* pNH 223, 2. Crude amidase extract, 3. Heat-treated amidase.

2.4. GENERAL DISCUSSION AND CONCLUSIONS

Cameron (2001) isolated and cloned the amidase gene into *E. coli* BL21 strain pNH 223 pLySs. His research work on the growth, expression and purification of the protein showed that the *E. coli* BL21 strain pLySs reaches stationary phase after approximately 390 minutes in a shake flask. In his results, the expression of the amidase using 0.4mM IPTG yielded a linear expression curve with a maximum specific activity of approximately 35.0U/mg pure protein [Cameron, 2001, PhD thesis]. In the present study, the culture reached stationary phase after approximately 300 minutes of growth in a BIOFLO 110 series fermentor, and in the absence of antifoam. The expression of the amidase yielded a profile with a maximum specific activity of approximately 20.0U/mg crude protein [section 2.3.2]. The faster growth of the strain in the BIOFLO 110 series fermentor could be attributed to high oxygen transfer rates and more favourable growth conditions as compared to a shake flask. Large volume fermentation of the recombinant *E. coli* BL21 strain encoding the amidase gene is vital if the enzyme is to be applied at industrial scale. 5L fermentation using the BIOFLO 110 series fermentor has shown that high levels of expression of the amidase could be achieved and that large-scale fermentation of the strain could be achieved even without the use of antifoam, although optimisation of fermentor operating parameters could facilitate improved growth.

The increase in pH of the growth media observed during fermentation [section 2.3.1] was attributed to the secretion of various proteins (e.g. β -lactamase) and other metabolic by-products, which are basic in nature. It is not known how growth of the recombinant *E. coli* BL21 strain and the expression of the amidase could be affected if the pH was to be controlled at the initial growth media pH (i.e. pH 6.8). Baek *et al.* (2003) maintained the pH of the growth media at 7.2 when producing a thermostable D-methionine amidase from *Brevibacillus borstelensis* BCS-1 in a 30L fermentor. The biomass yield was estimated at 7.5g (wet cells) per litre of broth [Baek *et al.*, 2003]. Conversely, 10L fermentation without controlling the pH of the thermophilic strain *Bacillus* spp. growth media resulted in a biomass yield of 0.55g (dry cells) per litre of broth. In section 2.3.1, it was reported that the biomass yield for the growth of the recombinant *E. coli* BL21 strain was approximately 0.5g (wet cells) per litre of broth. From these results, it is unclear whether pH control during fermentation actually affects growth and/or expression of the enzymes. Biomass yield is mainly a function of fermentation parameters such as pH, temperature and dissolved oxygen, and therefore in order to fully understand and optimize the biomass yield, further fermentation studies should be conducted taking into account all parameters that affect growth rate.

SDS-PAGE gel electrophoresis has confirmed from both the present studies [section 2.3.3] and earlier work by Cameron (2001) that the amidase has a subunit molecular weight of about 35kDa. In order to determine the native molecular weight of the amidase, the number of subunits must be determined using methods such as crystallization, gel permeation chromatography, and ND-PAGE. The molecular weight of the enantioselective amidase from *Brevibacillus borstelensis* BCS-1 was estimated to be 199kDa by gel permeation chromatography and 30kDa by sodium dodecyl sulphate/polyacrylamide gel electrophoresis [Baek *et al.*, 2003]. Therefore the enzyme was, in fact, a homo-hexamer of a single subunit. Komeda and Asano (2000) cloned, purified and characterized a monomeric D-stereospecific amino acid amidase from *Ochrobactrum anthropi* SV3 with a native molecular weight of about 40kDa, while Kim and Oriel (2000) cloned and expressed an amidase from a similar strain, *Bacillus* sp. BR449, and the enzyme was found to be approximately 38.6kDa in size.

Purification of enzymes usually involves heat-treatment (thermostable enzymes), ammonium sulphate, dialysis, and chromatography. Purification of the amidase studied in this report has showed that heat-treatment can purify the amidase to between 50 and 70% purity with purification folds of between 2.0 and 3.0. The purification step increased the total activity (U)

of the enzyme by about 200% [section 2.3.2]. For commercial applications, it could be possible to achieve more than 90% purity if heat-treatment was used in conjunction to other purification methods such as ammonium sulphate, dialysis, phenyl sepharose, and/or ion exchange chromatography. From these results, it can be concluded that heat-treatment is a good method for the purification of the amidase for research/laboratory purposes. Earlier studies of the amidase by Cameron (2001) have shown similar results, where a 2.8 fold purification and recovered purified amidase activity (U) of 123% were shown. These experiments were carried out in shake flasks, while a BIOFLO 110 series fermentor was used for the present study. More favourable expression conditions in a BIOFLO 110 fermentor may have resulted in a more active enzyme. The thermostable D-methionine amidase, studied by Baek *et al.* (2003), was purified 2.2-fold and recovered specific activity of 207% by ammonium sulphate precipitation. In summary, many amidases have been produced using recombinant strains. Purification of these enzymes has been achieved using various methods and in most cases the activity of the purified enzyme was enhanced. From the overall results, it can be concluded that growth, expression and purification of the amidase is a simple procedure, which could readily be scaled-up for industrial purposes.

Chapter 3 Primary characterization of the purified recombinant amidase from *Bacillus* sp. RAPc8

3.1. INTRODUCTION

In the previous chapter, results were reported on the growth, expression and purification of the amidase from *Bacillus* sp. RAPc8 cloned into *E. coli* BL21 strain pNH 223 pLySs. The recombinant *E. coli* strain was grown successfully, and the expressed amidase protein was extracted and purified. The novel amidase had unconfirmed biochemical properties, although Cameron (2001) briefly studied the properties in terms of pH, temperature and thermostability. Therefore, the main aim of the work reported in this chapter was to study the primary biochemical characteristics of the purified amidase.

An industrially useful enzyme must meet certain operating standards in order to minimize the production costs of the biocatalyst. Industrial processes operate under fairly harsh conditions and therefore a good industrial biocatalyst must be stable and able to function under these harsh conditions. Industrial reactors, whether whole cell, immobilized-enzyme or solid-catalytic, are often associated with high fluctuations in operating parameters including temperature, pH, and pressure. Thus, a catalyst or an enzyme that has restricted operating conditions could denature or deactivate when conditions become unsuitable. In the work reported in this chapter, the biochemical properties of the amidase, namely operating conditions (i.e. temperature, pH, and thermostability), substrate specificity, chiral selectivity and potential inhibition by various sulfhydryl reagents, chelating reagents, protease inhibitors, metal ions and co-solvents, were studied. The results were compared to similar work by Cameron (2001) and other literature reports on characterization of amidases.

3.2. MATERIAL AND METHODS

3.2.1. Materials

All chemicals were purchased from Sigma-Aldrich (SA), Aldrich-SA and Fluka except for L-leucinamide and L-alaninamide, which were purchased from Vitas-M labs. D-lactamide and L-lactamide were purchased from Sigma-Aldrich (international). All solvents were purchased from Merck-SA and amidase was prepared as described in Chapter 2.

3.2.2. Determination of amidase optimal reaction temperature

The reactions were carried out at temperatures between 30°C and 90°C, in 1.8ml Eppendorf tubes. 10µl of purified amidase extract (0.5mg/ml) was added to 300µl of 50mM phosphate buffer, pH 7.0, and the mixture was pre-incubated for 1 minute at the desired temperature. 25mM final concentration of acetamide (substrate) was then added and the reaction mixture was further incubated for 1 minute at the desired temperature. Control reactions were carried out with the absence of enzyme. Unless otherwise specified, all the reactions described in this chapter were assayed for amidase activity (or ammonia concentration) using the method described in section 2.2.7.

3.2.3. Determination of amidase optimal working pH

50mM sodium acetate, potassium phosphate and *tris*-HCl buffers were used for pH values between 4-5, 6-8, and 9-10 respectively. 10µl of purified amidase extract (0.5mg/ml) was added to 300µl of 50mM buffer of desired pH. The mixture was pre-incubated at 50°C for 1 minute. 25mM final concentration of acetamide was then added and the reaction mixture incubated for a further 1 minute at 50°C. The concentration of ammonia was determined using the method described in section 2.2.7. Control reactions were carried out with the absence of enzyme.

3.2.4. Determination of amidase thermal stability

The thermal stability of the amidase was determined at temperatures between 50°C and 80°C. An aliquot of purified amidase extract (1.0ml in an Eppendorf tube) was incubated in a water bath for 3 hours at a desired temperature. 10µl enzyme samples were withdrawn every 30 minutes. The samples were added to 300µl of 50mM phosphate buffer, pH 7.0, and then pre-incubated for 5 minutes at 50°C. 25mM final concentration of acetamide was then added and the reaction mixture was incubated for a further 5 minutes. The activity of the amidase was determined using the method described in section 2.2.7. Control reactions were carried out with the absence of enzyme.

3.2.5. Investigation of amidase acyl transfer activity

Acyl transfer reactions were investigated using a modified version of the method of Fournand *et al.* (1998). The reactions were carried out at 50°C in glass reaction tubes. 3.0ml of phosphate buffer, pH 7.0, was mixed with 25mM acetamide and 0.5M hydroxylamine solution. The mixture was pre-heated for 15 minutes at 50°C. 100µl of purified amidase extract was then added and the reaction mixture was incubated for a further 30 minutes. 500µl samples were withdrawn after 30 minutes of incubation, and mixed with 1.0ml of an acidic FeCl₃ solution (356mM FeCl₃ in 0.65M HCl). The formation of hydroxamic acids was assayed spectrophotometrically at 500nm. The concentration of the hydroxamic acid formed was determined using molar extinction coefficients (ϵ_M) adapted from Fournand *et al.* (1997). Control reactions were carried out with the absence of enzyme.

3.2.6. Determination of amidase substrate specificity

Initial stocks of 1M amides were prepared in deionised water, except that 1M L-alaninamide hydrochloride was prepared in 29v/v% NaOH, 1M D,L-phenylalaninamide was prepared in 1M NaOH, and 1M hexanoamide was prepared in 50v/v% ethanol. All the reactions were carried out in glass reaction tubes at 50°C. 100µl of purified amidase extract was added to 3.0ml of 50mM phosphate buffer, pH 7.0, and the mixture was pre-incubated for 5 minutes. 10mM final concentration of substrate (amide) was then added and the reaction mixture was incubated for a further 60 minutes. Samples were withdrawn after 30 min and 60 minutes of incubation, and assayed for amidase activity using the method described in section 2.2.7.

3.2.7. Determination of chiral selectivity of the amidase

Initial stocks of 1M D,L-lactamide, L-lactamide and D-lactamide were prepared in deionised water. The reactions were carried out in glass reaction tubes at 50°C. 100µl of purified amidase was mixed with 3.0ml of 50mM phosphate buffer, pH 7.0, and then pre-incubated for 5 minutes at 50°C. 10mM final concentration of the lactamide was then added, and the reaction mixture was incubated for a further 60 minutes. 100µl samples were withdrawn after 30 and 60 minutes of incubation, and assayed for amidase activity as described in section 2.2.7. Control reactions were conducted with the absence of enzyme.

3.2.8. Investigation of amidase potential inhibition

3.2.8.1. The effect of metal ions on amidase activity

Initial 1M stocks of various chlorides, nitrates and sulphates were prepared in deionised water. The reactions were carried out in glass reaction tubes at 50°C. 100µl of purified amidase was mixed with 3.0ml of 50mM phosphate buffer, pH 7.0, and then pre-incubated for 1 minute. 1mM final concentration of a metal ion was then added and the mixture was again pre-incubated for 15 minutes. 10mM final concentration of amide was then added and the reaction was carried out for 15 minutes. 100µl samples were withdrawn and assayed for amidase activity as described in section 2.2.7. Control reactions were conducted with the absence of enzyme, while standard reactions were conducted with absence of the metal ions.

3.2.8.2. The effect of organic co-solvent on amidase activity

Similar to section 3.2.7.1 above, 100µl of purified amidase extract was added to 3.0ml of 50mM phosphate buffer, pH 7.0, and the mixture was pre-incubated for 1 minute at 50°C. Organic co-solvent at 5v/v% and 10v/v% final concentration was then added and the mixture was further pre-incubated for 15 minutes. 10mM final concentration of amide was then added and the reaction was carried out for 15 minutes. 100µl samples were withdrawn and assayed for amidase activity as described in section 2.2.7. Control reactions were conducted with the absence of enzyme, while standard reactions were conducted with absence of the organic co-solvents.

3.2.8.3. The effect of sulfhydryl, chelating and protease inhibitors on amidase activity

Initial 100mM stocks of sulfhydryl (i.e. mercury chloride and hydrogen peroxide) and chelating reagents (i.e. EDTA and α,α' – dipyridyl) were prepared in deionised water while an initial 100mM stock of the protease inhibitor, PMSF, was prepared in anhydrous ethanol. The reaction were carried out at 50°C in glass reaction tubes. 100 μ l of purified amidase extract was mixed with 3.0ml of 50mM phosphate buffer, pH 7.0, and the mixture was pre-incubated for 1 minute. 1mM final concentration of an inhibitor (sulfhydryl, chelating or protease) was then added and the mixture was further pre-incubated for 15 minutes. Finally, 10mM final concentration of acetamide was added and the reaction was carried out for 15 minutes at 50°C. 100 μ l samples were withdrawn and assayed for amidase activity as described in section 2.2.7. Control reactions were conducted with the absence of enzyme, while standard reactions were conducted with absence of the chemical inhibitors.

3.3. RESULTS**3.3.1. Determination of amidase optimal reaction temperature**

The activity of the amidase towards acetamide was investigated across a range of reaction temperatures, according to section 3.2.2. The specific activity of the amidase was found to increase with increasing temperature until it reached a maximum at 50°C. Above 50°C the specific activity decreased with increasing temperature [Figure 3.1].

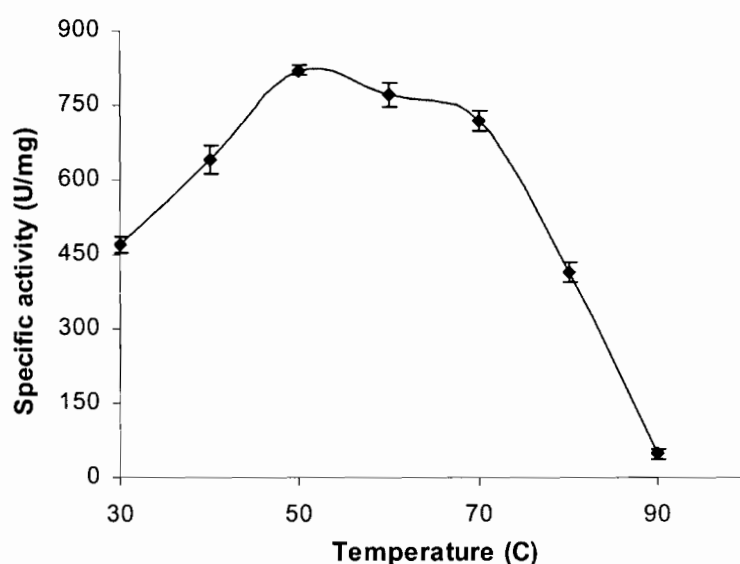


Figure 3.1: Specific activity of the *Bacillus* sp. RAPc8 amidase at different reaction temperatures.

3.3.2. Determination of *Bacillus* sp. RAPc8 amidase optimal working pH

Tris-HCl, phosphate and acetate buffers were used to vary the reaction pH of the amidase-catalyzed hydrolytic conversion of acetamide and acrylamide. Other reaction parameters were kept constant (viz., temperature, substrate concentration and total reaction volume), and the specific activity of the amidase was determined in the different pH solutions. At 25°C and atmospheric pressure, acetate buffer has a pKa value of 4.4 and good buffering capabilities at acidic pHs, phosphate buffer has a pKa value of 6.86 and good buffering capabilities around pH 5.6 - 8.6, while *tris*-HCl has a pKa value of 8.1 and good buffering capabilities under basic pH conditions. Therefore these buffers were used to cover the full range of pH values between 4.0 and 10.0. A good buffer for a biocatalytic reaction should have an ionic strength or pKa value close to the optimum working pH of the biocatalyst.

The specific activity was found to increase with increasing pH until it reached a maximum at pH 7.0, when both acrylamide and acetamide were used as substrates. Above pH 7.0 the specific activity decreased with increasing pH [Figure 3.2]. Phosphate buffer was used to obtain the results shown by figure 3.2, but the amidase showed no activity when acetate and *tris*-HCl buffer were used for pH ranges 4.0 - 5.0 and 9.0 - 10.0 respectively [results not shown]. The lack of activity observed in the amidase when *tris*-HCl and acetate buffers were

used may be due to the physical properties of the amidase of acetate and *tris*-HCl buffers. The results shown by figure 3.2 suggest that the amidase has an optimal working pH of 7.0.

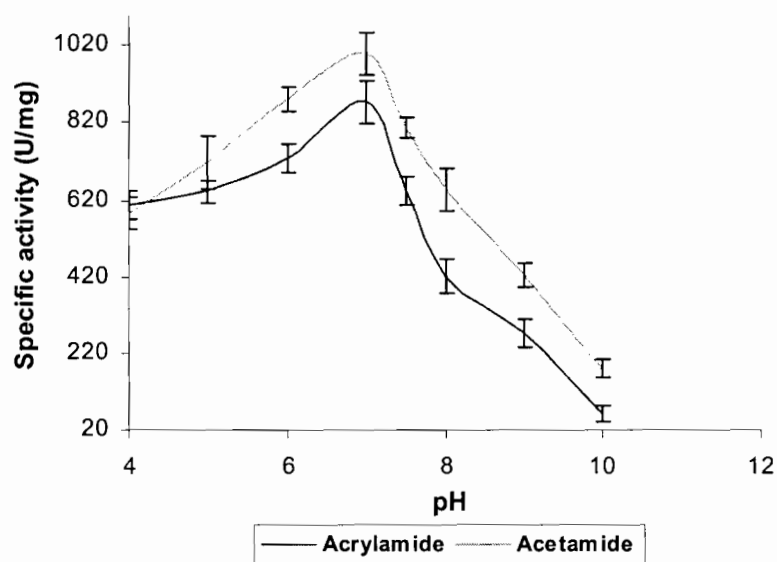
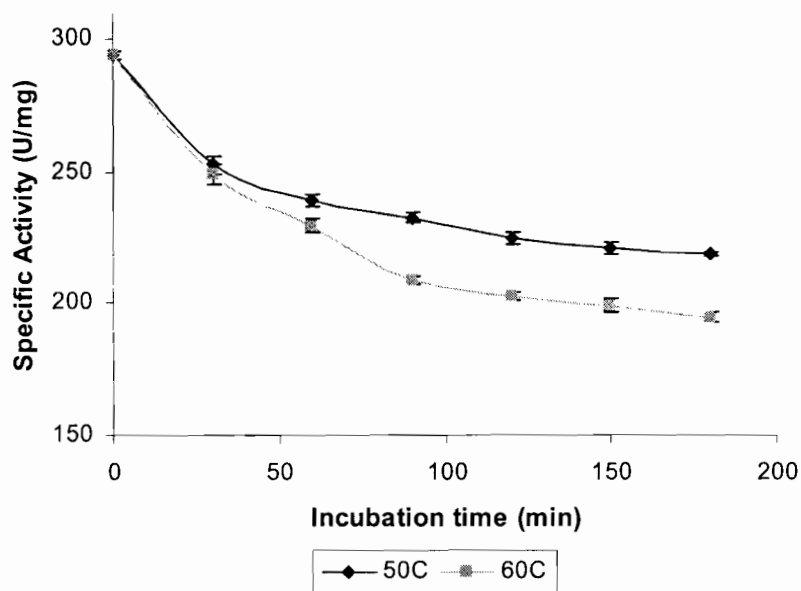


Figure 3.2: Specific activity of amidase in phosphate buffer at different pH values, using acrylamide or acetamide as substrates.

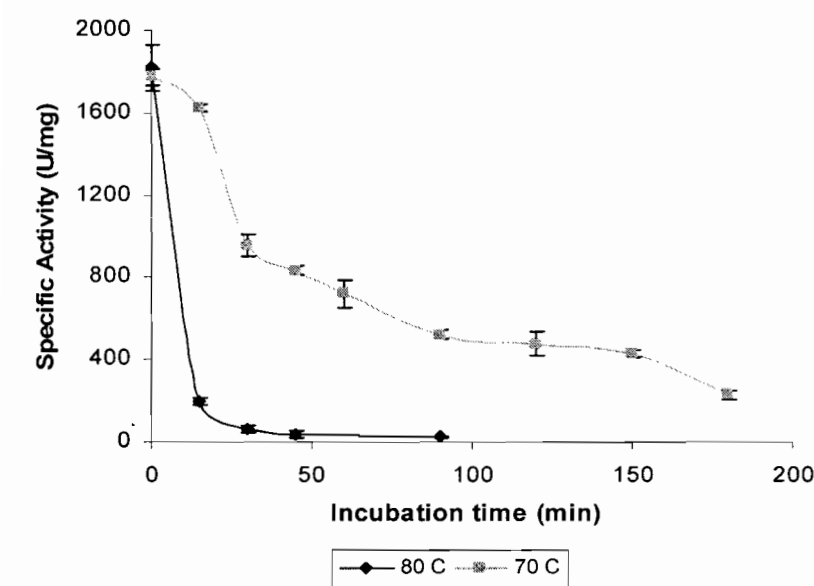
3.3.3. Determination of amidase thermal stability

Purified extracts of the amidase were incubated at various temperatures for long periods of time (approximately 3 hours). Samples were withdrawn periodically and used to determine the specific activity of the amidase towards acetamide and hence the thermal stability of the enzyme. The amidase showed high thermal stability at 50°C and 60°C respectively, in that the enzyme maintained high activity within the experimental time frame at these temperatures. The enzyme initially gave a specific activity of 290U/mg and after 3 hours of incubation the remaining specific activities were 218 and 195U/mg at 50°C and 60°C respectively [Figure 3.3]. This was an overall loss in specific activity of approximately 25% and 33% at 50°C and 60°C respectively, over 3 hours.



Figures 3.3: Specific activity of the amidase over 180 min incubation at 50 and 60°C.

The amidase showed significant loss of specific activity at 70°C and 80°C. At 70°C, the specific activity of the amidase decreased rapidly with incubation time, from an initial 1800U/mg to approximately 228U/mg in 3 hours while at 80°C, the specific activity dropped from an initial 1800U/mg to about 23U/mg in just 15 minutes of incubation [Figure 3.4]. This was an overall loss in specific activity of about 87% in 3 hours, and 98% in 15 minutes, at 70°C and 80°C respectively. The half-life of the amidase is defined as the time taken for the enzyme to lose half of its initial activity. The amidase maintained more than half its initial activity at 50°C and 60°C over the experimental time-frame. At 70°C and 80°C, the half-life of the amidase was estimated at 45 minutes and 8 minutes respectively.



Figures 3.4: Specific activity of the amidase over 180 min incubation at 70 and 80°C.

3.3.4. Determination of the amidase substrate specificity

The substrate specificity of the amidase was determined by conducting reactions using various amides as substrates. Amides were chosen based on aromatic or aliphatic nature, molecular size (short, medium or long chain), and the presence functional groups near the amide-bearing carbon (the α -carbon). The amidase was found to be highly active on short-chain aliphatic amides such as acrylamide, propionamide and acetamide; their relative activities were 447, 342 and 435% respectively, where the specific activity of the amidase towards formamide was taken as 100% [Figure 3.5]. The amidase was also very active on substituted short-chain and mid-length aliphatic amides such as fluoroacetamide, formamide, diacetamide, lactamide and isobutyramide; the relative activities were as 176, 100, 61, 52 and 51% respectively [Figure 3.5]. The amidase showed no activity towards the long-chain aliphatic amide hexanoamide, the short-chain aliphatic amides urea and L-alaninamide, and any of the aromatic substrates, including benzamide, nicotinamide, isonicotinamide, L-prolinamide and D,L-phenylalaninamide. These substrates, especially aromatic amides, could either be acting as amidase inhibitors or be unable to fit the amidase active site.

Table 3.1 shows the chemical structures of all the amides used for this experiment. Based on the results shown by Figure 3.5 and the chemical structures of the amides, the following

deductions can be made. The presence of a double-bond near the hydrolyzing group had no effect on the rate of hydrolysis, while the presence of other functional groups (i.e. methyl and hydroxyl groups) decreased the rate of hydrolysis. This was demonstrated by the relatively low activities for isobutyramide and lactamide, 52% and 51% respectively as compared to 447% for acrylamide. The presence of an –NH₂ group near the hydrolyzing group greatly affected the rate of amide hydrolysis. This was the case for very low relative activities for L-alaninamide and urea, 11% and 2% respectively. It is also possible that the pure isomers (i.e. L-alaninamide) were not hydrolysed due to the chiral selectivity of the amidase.

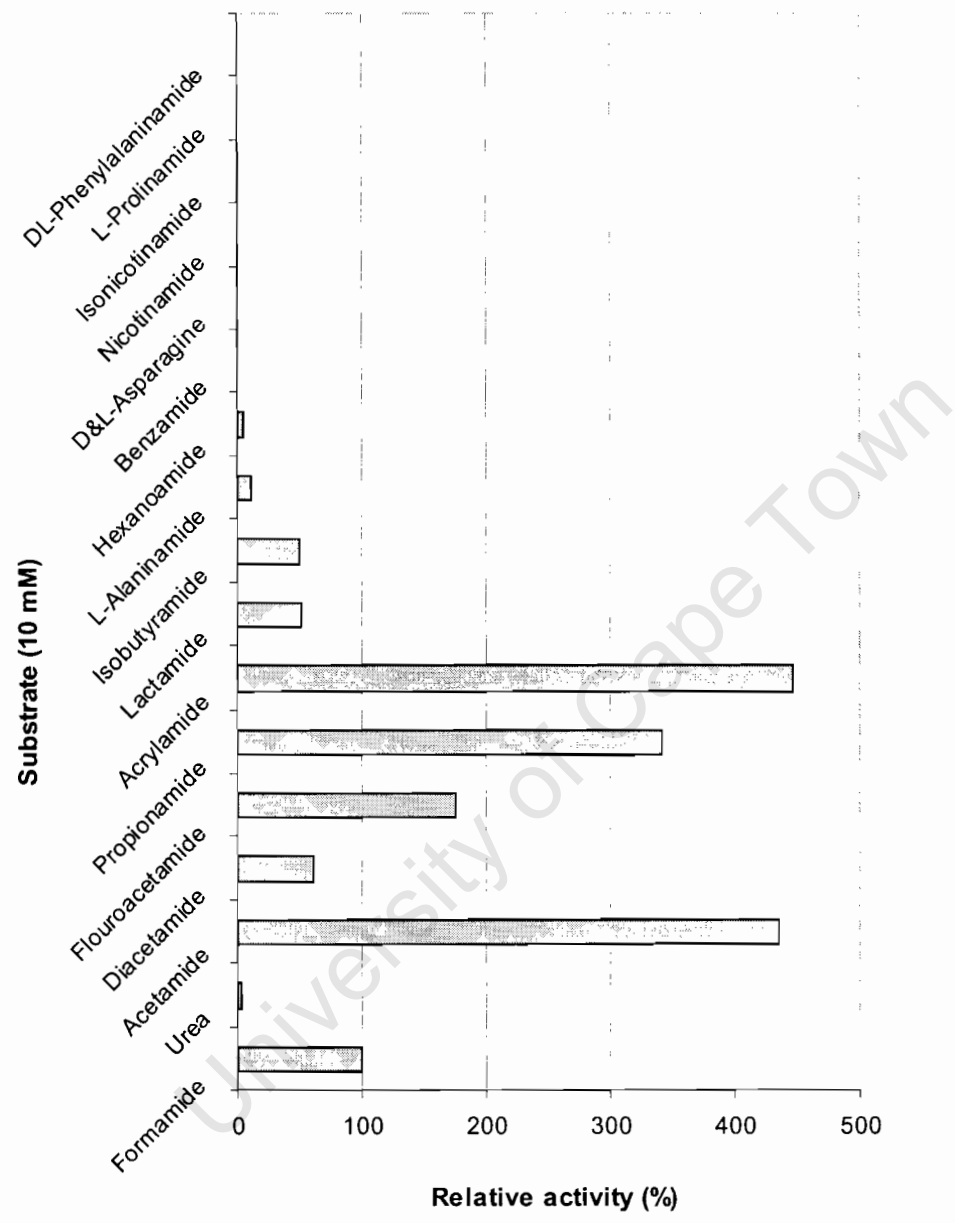
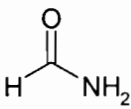
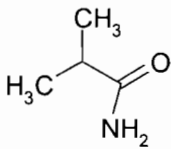
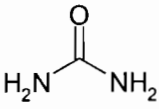
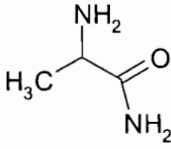
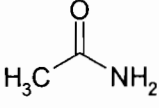
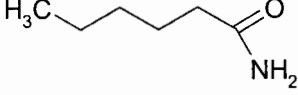
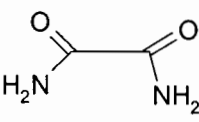
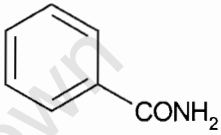
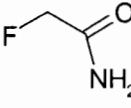
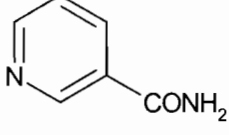
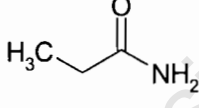
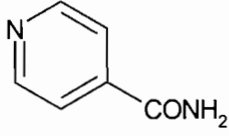
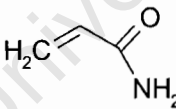
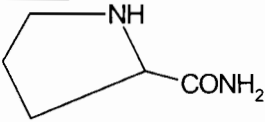
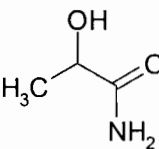
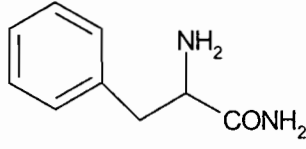


Figure 3.5: Relative activity of the *Bacillus* sp. RAPc8 amidase towards a range of different amide substrates.

Table 3.1: Chemical structures of amides used for the determination of amidase substrate specificity.

Chemical name	Chemical structure	Chemical name	Chemical structure
Formamide		Isobutyramide	
Urea		L-alaninamide	
Acetamide		Hexanoamide	
Diacetamide		Benzamide	
Flouroacetamide		Nicotinamide	
Propionamide		Isonicotinamide	
Acrylamide		L-prolinamide	
Lactamide		D,L-phenylalaninamide	

3.3.5. Determination of amidase chiral selectivity

Some enzymes are able to catalyse the conversion of the D-, L-, or both-isomers of a chemical compound. The substrate specificity of the amidase was limited to the hydrolysis of short- and mid-length aliphatic amides, as discussed in section 3.3.4. Lactamide, a short-chain amide, occurs as two isomers [Table 3.2], and was therefore used to determine the chiral selectivity of the amidase. Thus, amidase-catalyzed reactions using both racemic and pure isomers of the lactamide were conducted. Figure 3.6 shows the specific activity of the amidase towards D-lactamide, L-lactamide and D,L-lactamide respectively. The amidase was found to be more active on D-lactamide, least active on D,L-lactamide and not active on L-lactamide as substrates; the specific activity of the amidase on these substrates were estimated to be 16, 8 and 0% respectively. Therefore, the amidase was capable of hydrolyzing only the D-isomer of the lactamide.

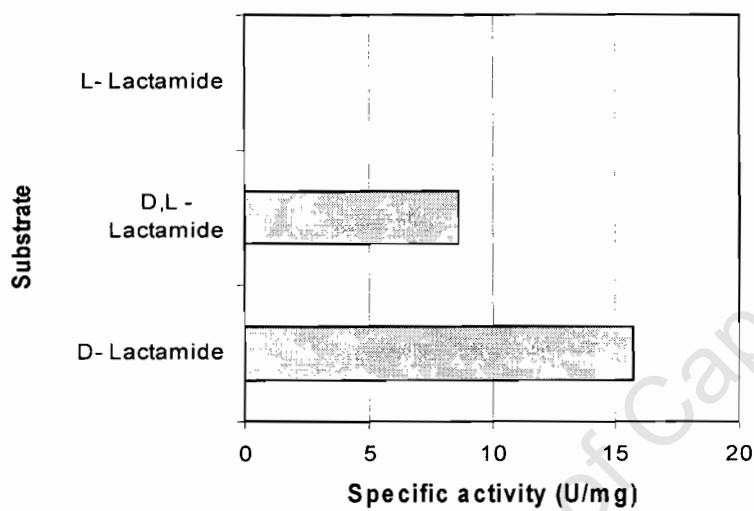


Figure 3.6: Chiral selectivity of the amidase towards lactamide.

Table 3.2: Chemical structures of lactamide isomers.

Chemical name	Chemical Structure
D-lactamide	
L-lactamide	

3.3.6. Investigation of amidase acyl transfer activity

Wide spectrum amidases have been reported to catalyse acyl transfer reactions, leading to the formation of hydroxamic acids. The reactions are conducted in the presence of excess acyl donor reagent. Amidase-catalysed reactions were conducted using various amides as substrates and hydroxylamine as an acyl donor reagent. As with hydrolysis reactions [section 3.3.4], in acyl transfer reactions, the amidase was more active towards short- and mid-length aliphatic amides such as acetamide, propionamide and isobutyramide; their relative activities were estimated as 100, 92.5 and 350% respectively [Figure 3.8]. The amidase was not active on long-chain aliphatic amides (hexanoamide), aromatic amides (benzamide, results not shown) and L-isomers of both short- and mid-length aliphatic amides (L-alaninamide and L-leucinamide) [Figure 3.8]. Control reactions were conducted with the absence of the biocatalyst, and rapid chemical reactions were observed with very short monoamides such as formamide, acrylamide and diacetamide, under the experimental conditions used. Fournand *et al.* (1997) also reported these spontaneous chemical reactions with short-chain amides such as formamide, acrylamide and diacetamide. Therefore, these compounds are not suitable for enzyme-catalyzed acyl transfer reactions. It is notable that the best substrate for acyl transfer activity was found to be isobutyramide, although the range of amides tested was not wide. The explanation for this behaviour is unknown, bearing in mind that for hydrolysis reactions discussed earlier, isobutyramide was not the most readily hydrolyzed substrate. As shown in Table 3.1, isobutyramide has a similar chemical structure to that of lactamide, with the difference being the presence of a methyl group on the α -carbon, in the case of isobutyramide, and a hydroxyl group in the case of lactamide. Therefore, perhaps the localization of electrons, due to these substitutions, could be the cause of this result. The acyl transfer reaction mechanism utilizing hydroxylamine as an acyl donor is shown in Figure 3.7.

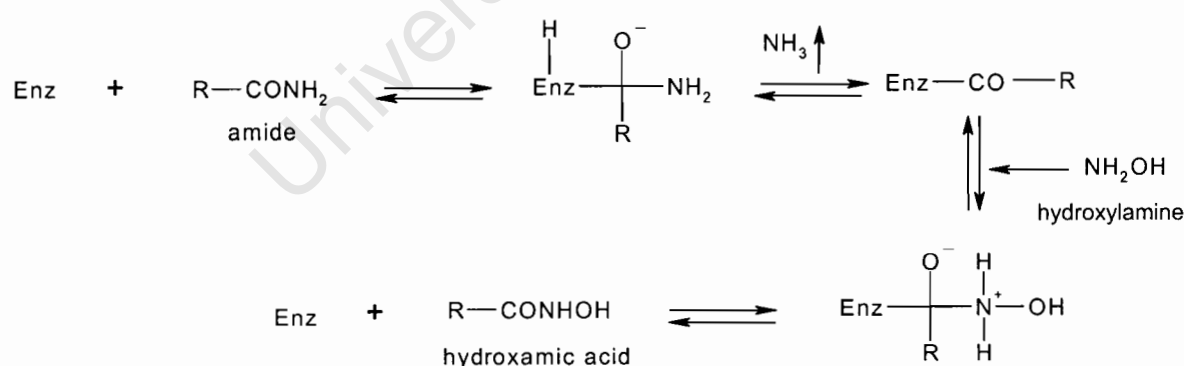


Figure 3.7: Amidase-catalyzed acyl transfer reaction mechanism (redrawn from Fournand *et al.*, 1997).

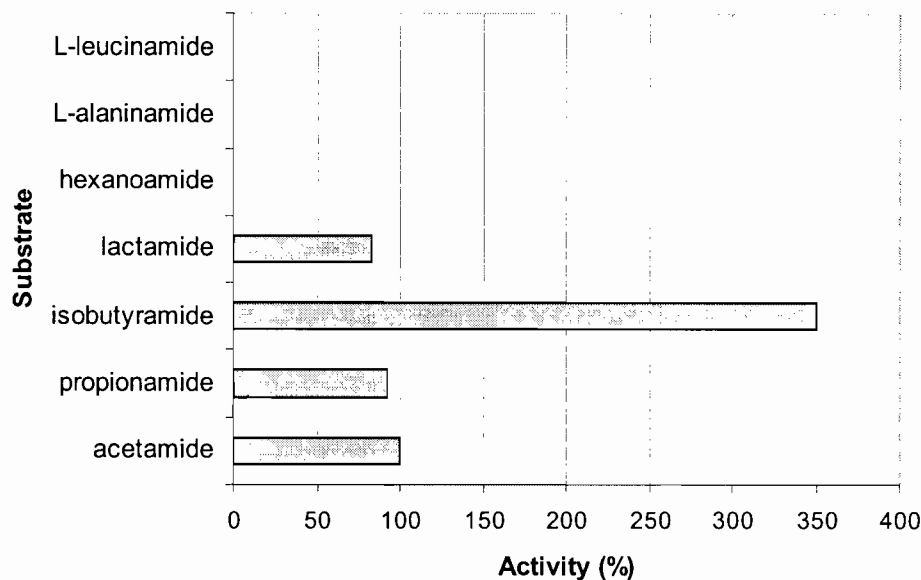


Figure 3.8: Relative acyl transfer activity of the *Bacillus* sp. RAPc8 amidase towards various substrates.

3.3.7. Investigation of potential amidase inhibition

The physical structure of an enzyme and the amino acid groups in its active site are susceptible to cleavage or change due to the presence reactive compounds in the reaction mixture. These compounds can alter the functionality or activity of the enzyme by deforming its physical structure or by reacting with catalytic residues at the active site. In this section, the investigation of the susceptibility of the amidase towards various reactive compounds (sulfhydryl, chelating and protease inhibitors) is described.

3.3.7.1. The effect of metal ions on amidase activity

Some metal ions are known to alter catalytic active-site residues, usually of non-metal dependent enzymes. The catalytic activity of the amidase towards acetamide, in the presence of various metal-containing compounds, was investigated. Heavy metals (typical sulfhydryl reagents) such as Cu^{2+} , Co^{2+} and Fe^{3+} were found to severely affect the specific activity of the amidase [Table 3.3]. The presence of other metal ions including Mg^{2+} , Zn^{2+} , Ba^{2+} and Mn^{2+} decreased the activity of the amidase by 60, 48, 41, and 12% respectively [Table 3.3]. Alkaline metal chlorides and nitrates such as NaCl, KCl, NaNO_3 and KNO_3 had almost no

effect on the activity of the amidase. Hydroxyl groups, present as NaOH and KOH, also had no significant effect on the activity of the amidase

Table 3.3: Specific activity of amidase in the presence of metal ions as potential inhibitors.

Metal ions	Concentration (mM)	% Remaining Activity ^a
Chlorides:		
NaCl	1.0	100
KCl	1.0	96
ZnCl ₂	1.0	64
BaCl ₂ .2H ₂ O	1.0	59
FeCl ₃	1.0	32
CoCl ₂ .6H ₂ O	1.0	28
Nitrates:		
NaNO ₃	1.0	92
KNO ₃	1.0	96
Sulphates:		
CuSO ₄ .5H ₂ O	1.0	11
MgSO ₄	1.0	40
ZnSO ₄ .7H ₂ O	1.0	52
MnSO ₄ .H ₂ O	1.0	88
Hydroxides:		
KOH	1.0	99
NaOH	1.0	100

^a Remaining activity after 15 minutes incubation in the presence metal ion was determined as described in section 3.2.3. The activity without metal ion was taken as 100%

3.3.7.2. The effect of organic co-solvent on amidase activity

The use of enzymes in organic solvents is important, mainly for reactions requiring increased substrate solubility or low water activity. Also, products and reactants can easily be separated in the case of reactions carried-out in non-miscible organic solvents as compared to aqueous solutions, due to the high volatility of most organic solvents as compared to water. In the work described in this section, amidase-catalyzed reactions were conducted in the presence of

various water-miscible organic solvents (co-solvents). All the co-solvents, tested at 5v/v% concentration, inhibited the amidase to varying degrees [Table 3.4]. Alcohols including methanol, ethanol, n-butanol and iso-propanol, decreased the activity of the amidase by 50, 58, 48 and 60% respectively; acetone and acetonitrile decreased the activity of the enzyme by 86% and 93% respectively, while DMS, DMSO and 2-Mercapto-ethanol completely inhibited the enzyme. In a further experiment, the amidase was completely inhibited by all these co-solvents, when tested at 10v/v% concentration.

Table 3.4: Specific activity of *Bacillus* sp. RAPc8 amidase in the presence of various organic co-solvents.

Co-solvent	Activity (U/mg)	% Remaining Activity ^a
None	83.0	100
2-Mercapto ethanol	0.0	0
Acetone	11.3	14
Acetonitrile	5.8	7
DMS	0.0	0
DMSO	0.0	0
Ethanol	34.7	42
Iso-propanol	12.1	30
Methanol	41.6	50
n-Butanol	43.0	52

Abbreviations: DMS, dimethylsulphide; DMSO, dimethylsulphoxide

^a Remaining activity after 15 minutes incubation in the presence of 5 v/v% co-solvent was determined as described in section 3.2.4. The activity without co-solvent was taken as 100%.

3.3.7.3. Effect of sulfhydryl, chelating and protease inhibitors on amidase activity

In this section, amidase-catalyzed reactions were conducted, in the presence of various chemical inhibitors. These chemical inhibitors, like organic solvents and metal ions, can change the physical structure of the enzyme or react with amino acid residues on the active site of the enzyme. The amidase was completely inhibited by the presence of sulfhydryl reagents including H₂O₂ and HgCl₂ [Table 3.5]. This finding agrees with the earlier discussion [section 3.3.7.1] that typical sulfhydryl-binding heavy metals severely inhibit the activity of the amidase. The presence of metal-chelating reagents such as EDTA and α,α' -

dipyridyl had no effect on the activity of the amidase, which suggests that this enzyme is not a metal-dependent enzyme. The presence of protease inhibitors (i.e. PMSF) also had no effect on the activity of the amidase and the presence of DTT increased the activity of the amidase by approximately 1.5 fold [Table 3.5].

Table 3.5: Remaining activity of amidase in the presence of various inhibitors.

Inhibitor	Concentration (mM)	% Remaining Activity ^a
Sulphydryl reagents		
H ₂ O ₂	1.0	1
HgCl ₂	1.0	5
Chelating reagents		
None	1.0	100
EDTA	1.0	98
α,α' - dipyridyl	1.0	99
DTT	1.0	149
Protease inhibitors		
None	1.0	100
PMSF	1.0	98

Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol

^a Remaining activity after 15 minutes incubation in the presence of an inhibitor as described in section 3.2.5. The activity without an inhibitor was 100%

3.4. GENERAL DISCUSSION AND CONCLUSIONS

Presently, relatively few thermostable microorganisms encoding nitrile-hydrolyzing enzymes (i.e. nitrile hydratases, nitrilases, and amidases) have been isolated, and their enzymes purified and characterized [Chapter 1]. After isolation of a new enzyme, its physical and biochemical characteristics should be determined prior to utilizing it in biocatalytic process. Preliminary characterization of the amidase from *Bacillus* sp. RAPc8 by Cameron (2001) had

shown that the optimal operating conditions of the amidase were 50°C and pH 7.2. In the present study, primary characterization of the amidase based on reaction temperature and pH [section 3.3.1-3.3.2] has shown results identical to those reported by Cameron (2001), and therefore it can be concluded that the amidase maintains the highest specific activity under these conditions. The amidase, studied in this report, exhibited optimal working conditions at 50°C and pH 7.0, and was thermally stable between temperatures 50 and 70°C. In comparison to literature reports by others, the amidase was found to have characteristics similar to the same class of other amidases, including the aliphatic amidases from *Rhodococcus rhodochrous* M8 [Kotlova *et al.*, 1999], *Rhodococcus* sp. [Nawaz *et al.*, 1994], *Bacillus stearothermophilus* BR388 [Cheong and Oriel, 2000], *Pseudomonas chlororaphis* B23 [Ciskanik *et al.*, 1995], *Klebsiella pneumoniae* NCTR1 and *Arthrobacter* sp. J1 [Asano *et al.*, 1982]. These enzymes have optimal working conditions between 35 and 65°C, and pH 7.0 to 8.6.

Studies of the substrate specificity and chiral selectivity of the amidase [section 3.3.4 – 3.3.5] have shown that the amidase from *Bacillus* sp. RAPc8 is not capable of hydrolyzing aromatic amides or long-chain aliphatic amides. The aliphatic amidases from *Pseudomonas aeruginosa* [Clarke, 1970], *Rhodococcus* sp. R312 [Maestracci *et al.*, 1984; Soubrier *et al.*, 1992], *Arthrobacter* sp. J1 [Asano *et al.*, 1982], *Methylophilus methylotrophus* [Wyborn *et al.*, 1996], *Helicobacter pylori* [Skouloubris *et al.*, 1997] and *Bacillus stearothermophilus* BR388 [Cheong and Oriel, 2000] have similar properties, and belong to the group of wide spectrum aliphatic amidases. Therefore, the substrate specificity of the amidase from *Bacillus* sp. RAPc8 suggest that the enzyme also belong to the group of wide spectrum aliphatic amidases, which catalyze the hydrolysis of only short- and mid-length aliphatic amides, and where binding of substrate on the active site is affected by the presence –NH₂ substitutions near the hydrolyzing group [Fournand and Arnaud, 2001]. Furthermore, substrates such acetamide, acrylamide and propionamide are generally the most rapidly hydrolyzed amides [Fournand and Arnaud, 2001]. Table 3.6 summarises some thermostable amidases reported by others, their optimum working conditions and substrate selectivity.

The amidase from *Bacillus* sp. RAPc8 demonstrated the ability to catalyze acyl transfer reactions in the presence of hydroxylamine, as outlined in section 3.3.6. This result supports the findings by Fournand *et al.* (1998) that all aliphatic amidases are capable of catalyzing these reactions. Fournand *et al.* (1998) studied the acyl transfer activity of a recombinant

amidase from *Rhodococcus* sp. R312 overproduced in *Escherichia coli* strain, and found that the enzyme was more active with substrates such acetamide, propionamide and acrylamide. Similarly, the amidase studied in this report was more active on acetamide, propionamide and isobutyramide [section 3.3.6].

Table 3.6: A summary of some thermostable amidases reported in literature.

Microorganisms	Optimum working pH and temperature	Substrate selectivity	Reference
<i>Klebsiella pneumoniae</i> NCTR1	7.0, 65°C	Aliphatic amides	[Nawaz <i>et al.</i> , 1996]
<i>Pseudomonas Chororaphis</i> B23	7.0 – 8.6, 50°C	Aliphatic & aromatic amides	[Ciskainik <i>et al.</i> , 1995]
<i>Bacillus stearothermophilus</i> BR388	7.0, 55°C	Wide spectrum amidase	[Cheong and Oriel, 2000]
<i>Rhodococcus erythropolis</i> MP50	7.5, 55°C	Aromatic amides	[Hirrlinger <i>et al.</i> , 1996]
<i>Rhodococcus rhodochrous</i> M8	7.0, 55-60°C	Aliphatic amides	[Kotlova <i>et al.</i> , 1999]

The second group of aliphatic amidases, which includes amidases able to hydrolyze mid-length amides, arylamides, α -aminoamides and α -hydroxyamides have been reported to be enantioselective towards several racemic amides [Chebrou *et al.*, 1996]. In section 3.3.5, the activity of the amidase from *Bacillus* sp RAPc8 towards the pure isomers and a racemic mixture of lactamide were discussed. The specific activities of the amidase towards D-lactamide, D,L-lactamide and L-lactamide were estimated as 16, 8 and 0% respectively. These findings suggest that the amidase was D-specific towards the lactamide and thus, it can be concluded that the amidase possesses enantioselectivity towards lactamide. A number of other D-specific amidases have been reported in literature, including the thermostable D-methionine amidase from *Brevibacillus borstelensis* [Baek *et al.*, 2003], and a D-amino acid amidase isolated from soil samples [Hongpattarakere *et al.*, 2003]. D-specific amidases can be

used for the kinetic resolution and production of D-amino acids, and the production of optically pure carboxylic acids. Other enantioselective amidases reported in literature includes the amidases from *Rhodococcus* sp. R312 [Mayaux *et al.*, 1990], *Rhodococcus* sp. N-774 [Hashimoto *et al.*, 1991], *R. erythropolis* JCM6823 [Duran *et al.*, 1993], *R. rhodochrous* J1 [Kobayashi *et al.*, 1993], *R. erythropolis* MP50 [Hirrlinger *et al.*, 1996], *Ps. Chlororaphis* B23 [Ciskanik *et al.*, 1995] and *Bacillus* sp. BR449 [Kim and Oriel, 2000]. In this class of aliphatic amidases, substrates such as valeramide, hexanoamide and adipamide are generally the most rapidly hydrolyzed amides [Fournand and Arnaud, 2001].

In general, amidases are not metal-dependent enzymes and do not contain metal co-factors in their active site. Inhibition of the amidase from *Bacillus* sp. RAPc8 by heavy metals ions such as Hg^{2+} , Fe^{2+} and Co^{2+} supports the suggestion that these enzymes are not metal-dependent [section 3.3.7.1]. The presence of DTT in the reaction mixture resulted in an approximately 1.5-fold increase in amidase activity [section 3.3.7.3], which suggests the important role of sulfhydryl groups in the mechanism of action of the enzyme. With the exception of the amidases from *Corynebacterium* sp. C5 and *Stenotrophomonas maltophilia*, amidases are generally thought to have sulfhydryl groups which are essential for catalysis [Kotlova *et al.*, 1999].

Due to the hydrophobic nature of many substrates, organic co-solvents are often added to the reaction medium containing biocatalysts. The sensitivity of nitrile-degrading enzymes towards co-solvents has only allowed the use of low percentages of the co-solvents (5-10v/v %) [Mylerova and Martinkova, 2003]. The amidase studied in this report was no exception, as the presence of 5v/v% co-solvents, including ethanol, methanol and n-butanol, resulted in 42, 50 and 52% loss of amidase activity, respectively. Recent results reported by others have shown that both crude and purified enzymes can be used with high percentages of organic co-solvents present in the reaction medium; a purified nitrile hydratase from *Rhodococcus equi* was utilized in high hydrocarbon concentration 2-phase systems with up to 90 v/v% isooctane or pristine [Mylerova and Martinkova, 2003]. Also, several studies on the application of amidases in low-water media have been reported, including studies of a peptide-amidase catalyzing C-terminal peptide amidation in an acetonitrile medium, containing 25v/v% dimethyl formamide and 3v/v% water [Čeřovský and Kula, 2001]. Studies have also shown that methanol and ethanol are suitable co-solvents for purified enzymes, but only up to 20v/v% [Mylerova and Martinkova, 2003]. Therefore, enzymes are susceptible to high

percentages of water-miscible organic co-solvents, but can be used in media containing high percentages of non-miscible organic co-solvents.

Biocatalyst inhibition by various compounds is a factor that must be avoided when dealing with an industrial biocatalyst, and hence it is very important to study the type and class of compounds that negatively affect the activity of an enzyme towards the production of desired products. From the amidase inhibition results and discussion earlier [section 3.3.7], it can be concluded that heavy metals and sulfhydryl reagents, at concentrations as low as 1mM in the reaction mixture, severely affect the activity of the amidase. It can also be concluded that organic alcohols are partial inhibitors of the amidase activity while heterocyclic or sulphur-containing organic co-solvents including DMS and DMSO inhibit the amidase completely, at concentrations as low as 5v/v% in the reaction mixture.

Generally, nitrile-converting enzymes are known to be active in a narrow range of temperatures and pH values (neutral or slightly alkaline), under heavy metals-free conditions and in the presence of low percentages of co-solvents due their sensitivity [Mylerova and Martinkova, 2003]. The general trend in the primary characterization of the amidase studied in this report has shown similar results, and this general behaviour could be to disadvantageous in its industrial applications, in that operating parameters such as temperature and pH should be strictly monitored and controlled for unsuitable fluctuations. Therefore, this requirement is likely to increase the operating costs of the industrial process.

Chapter 4 Immobilization of the recombinant amidase with different techniques and primary characterization of the immobilized amidase

4.1. INTRODUCTION

The use of immobilized enzymes, pioneered in the 1960s, is one of the most useful methods for stabilization of biocatalysts [Katchalski-Katzir and Kraemer, 2000]. Immobilized enzymes, defined by Katchalski-Katzir, are 'enzymes physically confined or localized in a defined space with retention of their catalytic activities, and which can be used repeatedly and continuously'. Immobilization of enzymes often causes some loss of catalytic activity but higher stability of the enzyme, although it may also cause an increase in activity due to more favorable micro-environmental conditions [Katchalski-Katzir and Kraemer, 2000].

The disadvantages of immobilization are that:

- Losses of enzyme activity can occur during and after immobilization due unfavorable reaction conditions.
- Diffusion or partitioning of substrates and products may be hampered by confinement of the enzyme inside the immobilized layer.
- The enzyme may have a more constrained conformation in the immobilized state, giving it a lower catalytic activity.
- There may be a high initial investment for the immobilization, compared to use of free enzyme.

The advantages of immobilization are that:

- Prevention of losses due to flushing away of enzyme is possible.
- A more stable biocatalyst may be obtained.
- There is the possibility of producing a biocatalyst with altered properties such thermostability and substrate specificity.

4.1.1. Stabilization of a biocatalyst by immobilization

In the work described in previous chapters, conditions were optimized for biocatalytic activity and the primary characteristics of the amidase from *Bacillus* sp. RAPc8, were determined. In the work reported in this chapter, the objective was to investigate methods of immobilizing and stabilizing the amidase, and thereby develop a biocatalyst for potential industrial application. The stability of the biocatalyst and cost are very important criteria for the choice and industrial application of enzymes [Martin *et al.*, 2003]. A number of modern immobilization techniques have been studied and developed, including encapsulation of the enzyme into a compartment inside a semi-permeable (porous) membrane, adsorption to various surfaces, covalent binding onto various support material [Boshoff *et al.*, 2003] and cross-linking of enzyme molecules.

4.1.2. Immobilization strategies

The choice of a suitable immobilization technique is based on, amongst other properties, the physical properties (i.e. strength, compressibility, surface area, porosity, permeability and etc.) of the support material, the chemical properties (i.e. hydrophilicity, inertness and etc.) of the support material, and the stability and resistance to bacterial/fungal attack of the support material [Bickerstaff *et al.*, 1997]. The nature of ligands in the active site of the enzyme and the types of amino acid residues are also factors to be considered when choosing an immobilization technique. Immobilization often involves the formation of a bond between the support material and the ligands and/or amino acid residues, found on the surface of the enzyme. Therefore, the nature and type of ligands and amino acid residues will determine how the enzyme will bind and the effectiveness and strength of the binding.

In the present study, the anticipated reactor configuration was also considered to be a major factor in the choice of immobilization technique. A certain immobilization technique may only be suitable for a fluidized-bed reactor and not suitable at all for a fixed-bed reactor. For example, enzymes immobilized on membranes (e.g. nylon) can hardly be used in a fluidized-bed reactor system. Therefore, for cases in which a reactor is chosen prior to immobilization, this factor will definitely influence the choice of the immobilization method.

In the work reported in this chapter, purified extracts of the amidase from *Bacillus* sp. RAPc8 were immobilized by adsorption on ethylene diamine activated Amberlite-XAD57 beads cross-linked with glutaraldehyde, immobilized by covalent binding on Eupergit C beads at 4 and 25°C, immobilized by covalent binding on Eupergit C beads coupled with bovin serum albumin at room temperature, and immobilized by covalent binding on Eupergit C beads with subsequent cross-linking using glutaraldehyde at room temperature. Finally, crude extracts of the amidase were immobilized by entrapment in a polyacrylamide gel at room temperature.

4.2. MATERIALS AND METHODS

4.2.1. Materials

All chemical were purchased from Sigma-Aldrich (SA), Aldrich-SA and Fluka. All solvents were purchased from Merck-SA. Eupergit C and Eupergit CM acrylic beads were received as a gift from Röhm Pharma Polymers (Darmstadt, Germany). Amidase was prepared and purified according to the methods described in Chapter 2.

4.2.2. Amidase immobilization by covalent binding on Eupergit C beads at 4°C and 25°C

Purified amidase extracts were first concentrated to between 2.0 and 5.0mg/ml by dialysis using 40w/v% polyethylene glycol (PEG). 5ml (2mg/ml protein concentration) of the enzyme was added to 1g dry Eupergit C beads and the mixture was incubated at room temperature (~25°C) and 4°C for 72 hours. The immobilized beads were recovered by filtration and then washed three times with 15ml of 50mM phosphate buffer, pH 7.0. Immobilized beads were collected from the filter and the filtrates, supernatant and washings were collected separately into 50ml Falcon tubes. The amount of unbound protein was determined from the supernatant and the washings using the method described in section 2.2.6. Unless otherwise stated, the activity of the immobilized amidase was determined by incubating 200mg wet immobilized beads mixed with 3.0ml of 50mM phosphate buffer, pH 7.0 and 10mM Acetamide at 50°C for 60 minutes. The specific activity was determined using method described in section 2.2.7.

4.2.3. Amidase immobilization by covalent binding on Eupergit C beads, coupling with bovine serum albumin

This method was adapted from Torres-Bacete *et al.* (2000). 5ml (5mg/ml protein concentration) of a purified amidase extract was added to 1g dry Eupergit C beads. Phenoxyacetic acid (30mM) was added to the mixture as a competitive inhibitor, in order to avoid possible interaction between the oxirane group of the Eupergit C and the catalytic residues in the active site of the amidase during immobilization. The mixture was then incubated at room temperature for 24 hours.

Immobilized beads were recovered by filtration and were washed three times with 15ml of 50mM phosphate buffer, pH 7.0. The filtrates, supernatant and washings, were collected separately into 50ml Falcon tubes. The matrix of the immobilized beads was modified by the addition of 5.0ml (5mg/ml) BSA. The mixture was further incubated for 24 hours at room temperature. The modified immobilized beads were recovered by filtration and washed, and the filtrates collected as above. The amount of unbound protein was determined from both the supernatants and the washings.

4.2.4. Amidase immobilization by covalent binding on Eupergit C beads with subsequent cross-linking

A modification of the method described in section 4.2.2 was used for this immobilization. 5ml (5mg/ml) of purified amidase extract was added to 1g dry Eupergit C beads. The desired final concentration of glutaraldehyde and EDAC (varied between 2w/v% and 0.5w/v%) was added into the mixture and then gentle shaking was continued for approximately 5 minutes. Immobilization and recovery of immobilized beads was performed as outlined in section 4.2.2.

4.2.5. Amidase immobilization by entrapment in polyacrylamide gel

A method adapted from Toogood *et al.* (2002) was used for this immobilization. 30ml (1.2mg/ml protein concentration) of crude amidase extract was combined with 10ml of 30w/v% acrylamide:bisacrylamide (29:1) solution and 12.5ml of 0.5M *tris*-HCl pH 8.0. Polymerisation was initiated by the addition of 0.05ml TEMED and 0.5ml of 10w/v%

ammonium persulphate. The solution was allowed to polymerize by incubating for 16 hours at room temperature. The gel was crushed to an average of 2mm pieces, washed with 100ml of 50mM phosphate buffer, pH 7.0 and recovered by centrifugation at 8000rpm for 20 minutes at 4°C. The gel was dried overnight in a vacuum dessicator.

Unless otherwise stated, the activity of the immobilized amidase was determined by incubating 500mg immobilized gel particles with 3.0ml of 50mM phosphate buffer, pH 7.0 and 10mM acetamide at 50°C for 60 minutes.

4.2.6. Amidase immobilization by adsorption onto EDA-Amberlite-XAD57 beads

a) Preparation of beads

A method adapted from Toogood *et al.* (2002) was used for this immobilization. Amberlite-XAD57 beads (50grams) were refluxed with 30ml ethylene diamine and 80ml toluene at 120°C for 5 hours. EDA-Amberlite-XAD57 beads were then recovered by filtration, washed with 50ml absolute methanol and dried overnight in a vacuum dessicator. Glutaraldehyde activated EDA-Amberlite XAD57 beads were prepared by mixing 50grams of the beads with 100ml of a 2.5w/v% glutaraldehyde solution, and the mixture was shaken constantly overnight at room temperature. The beads were then recovered by filtration, washed twice with 50ml of 50mM phosphate buffer, pH 7.0 and dried in a vacuum dessicator. The beads were stored at room until further use.

b) Immobilization

5.0ml (2.0mg/ml protein concentration) of purified amidase extract was added to 1g dry activated EDA-Amberlite-XAD57 beads. The mixture was incubated for 72 to 96 hours at room temperature. Immobilized beads were recovered by filtration, washed three times with 15ml of 50mM phosphate buffer, pH 7.0, and stored at -20°C until further use. The filtrates, supernatant and washings, were collected in 50ml Falcon tubes and used to determined the amount of unbound protein.

4.2.7. Primary characterization of immobilized amidase

In the previous chapter (sections 3.2.2 to 3.2.4), purified free-amidase extracts were characterized in terms of reaction temperature, pH and thermal stability. In this section, the experiments were repeated using an immobilized amidase, using the methods described in sections 3.2.2 to 3.2.4, with the difference being the use of 200mg wet immobilized beads instead of free enzyme.

4.3. RESULTS

4.3.1. Immobilization of the *Bacillus* sp. RAPc8 amidase with different techniques

The results obtained from the immobilization of crude and purified amidase extracts with different immobilization techniques are shown in Table 4.1. The protein binding yield of the support and the recovered amidase activity are shown for each immobilization method, and were defined as the amount of protein (%) bound onto the support material and the specific activity of the immobilized enzyme relative to free enzyme (%) respectively. Immobilization of the amidase by covalent binding on Eupergit C beads, both at 4°C and 25°C, and adsorption of the amidase on activated Amberlite-XAD57 beads yielded low protein binding capacities. Less than 40% protein binding yield was obtained for both these immobilization methods [Table 4.1]. This result may have been due to unfavourable immobilization conditions (e.g. temperature), and inefficient activation of the Amberlite-XAD57 beads. Significantly higher protein binding yields, 56.6 and 59.6% respectively, were obtained from immobilization by covalent attachment onto Eupergit C beads coupled with BSA and covalent attachment on Eupergit C beads cross-linked with 0.5w/v% glutaraldehyde and EDAC. The results could be attributed to the fact that bovine serum albumin (BSA) increased the protein loading on the beads, while glutaraldehyde and EDAC formed cross-linked enzyme molecules, thus allowing them to “stick” together and form aggregates. Increasing protein loading allowed more protein molecules to bind on the support, while cross-linking allowed the formation of support-enzyme aggregates, thus decreasing the tendency for washing-off of enzyme molecules. Immobilization by covalent binding on Eupergit C beads cross-linked with 1.0 and 2.0w/v% glutaraldehyde and EDAC resulted in very high protein binding yields,

90.2 and 93.6% respectively. Thus the higher concentration of glutaraldehyde and EDAC gave a higher binding yield of the amidase.

In comparison with the initial activity of the amidase, low activities were obtained for the amidase after immobilization by covalent binding on Eupergit C beads (4°C), entrapment in a polyacrylamide gel, adsorption on Amberlite-XAD57 beads and covalent binding on Eupergit C beads cross-linked with 1.0 and 2.0w/v% glutaraldehyde and EDAC. The remaining activities, after immobilization by these methods, were estimated to be 28.2, 1.6, 17.8, 29.4 and 48.2% respectively [Table 4.1]. In the case of immobilization with subsequent cross-linking, high concentrations of glutaraldehyde and EDAC in the immobilization media could have been inhibitory to the enzyme, and could have lead to the formation of strongly cross-linked aggregates, resulting in very low substrate diffusion rates through the immobilized matrix. Entrapment of the amidase in a polyacrylamide gel involved the addition of high concentrations of acrylamide/bisacrylamide and the addition of ammonia persulphate, which may have resulted in inhibitory effects on the enzyme. Hence, very low amidase activity was recovered after immobilization. Unfavourable immobilization conditions (4°C) could have been the reason for low recovery of amidase activity immobilization by covalent binding onto Eupergit C beads, in that the low temperature could have facilitated slower reaction between amino acid residues of the amidase and oxirane groups of the Eupergit C beads.

Immobilization by covalent binding on Eupergit C beads at 25°C, coupling with BSA, and cross-linking with 0.5w/v% glutaraldehyde and EDAC, both resulted in high immobilized amidase activities. Favourable immobilization conditions (25°C) and less inhibitory effects by glutaraldehyde and EDAC can be attributed for these results [Table 4.1]. The best immobilization technique was regarded as the method that resulted in both the highest protein binding yield and recovered enzyme activity. Thus, covalent binding of the amidase on Eupergit C beads, cross-linking with 0.5w/v% glutaraldehyde and EDAC, was the best immobilization technique. The protein binding yield and recovered amidase activity after immobilization were 59.6 and 78.9% respectively.

Table 4.1: Binding yields and % recovered activities for the amidase immobilized with different techniques.

Immobilization method	Binding yield ^a (%)	Recovered activity ^b (%)
Eupergit C, 4°C	13.3	28.2
Eupergit C, 25 °C	35.4	94.2
Polyacrylamide gel, 25 °C	-	1.6
Eupergit C (BSA coupled), 25 °C	56.6	81.4
Eupergit C (2.0 w/v% Glut./EDAC)	93.6	29.4
Eupergit C (1.0 w/v% Glut./EDAC)	90.2	48.2
EDA-Amberlite XAD57 (Glut. Activated)	3.8	17.8
Eupergit C (0.5 w/v% Glut./EDAC)	59.6	78.9

Abbreviations: BSA, bovin serum albumin, EDAC, ethylenediaminetetraacetic acid, Glut., glutaraldehyde

^a binding yield is defined as the amount of protein bound on the support. This was determined by comparing protein concentrations between the initial sample and the filtrates/washings.

^b recovered activity was defined as the specific activity of immobilized protein as compared to the same amount of free protein. This was determined by comparing the specific activities of immobilized and free amidase.

4.3.2. Primary characterization of immobilized amidase

In the work reported in section 4.3.1 it was concluded that covalent binding of the amidase on Eupergit C beads cross-linked with 0.5w/v% glutaraldehyde and EDAC was the best immobilization technique, for purified amidase extracts. In this section, work is reported in which the amidase, immobilized using the technique, was characterized in terms of optimum reaction temperature, pH and thermal stability.

Earlier work by Graham *et al.* (2000) on nitrile biotransformation using free and immobilized cells of the *Bacillus* sp. RAPc8 strain in alginate beads has shown that the thermal and biochemical properties of the cells remained unchanged even after immobilization. Therefore, it would be interesting to see whether the biochemical properties of the amidase from the *Bacillus* sp. RAPc8 will change after covalent immobilization on Eupergit C beads.

4.3.2.1 Determination of optimum reaction temperature of the immobilized amidase

Amidase-catalyzed reactions using immobilized enzyme were conducted at a range of reaction temperatures. The specific activity of the immobilized amidase increased with increasing reaction temperature until a maximum at 50°C, and above this temperature, the specific activity decreased with increasing reaction temperature. In comparison with the

results obtained using free amidase [section 3.3.1], it can be noted that above 50°C reaction temperature, the specific activity of the immobilized amidase decreased more slowly than the activity of the free amidase [Figure 4.1].

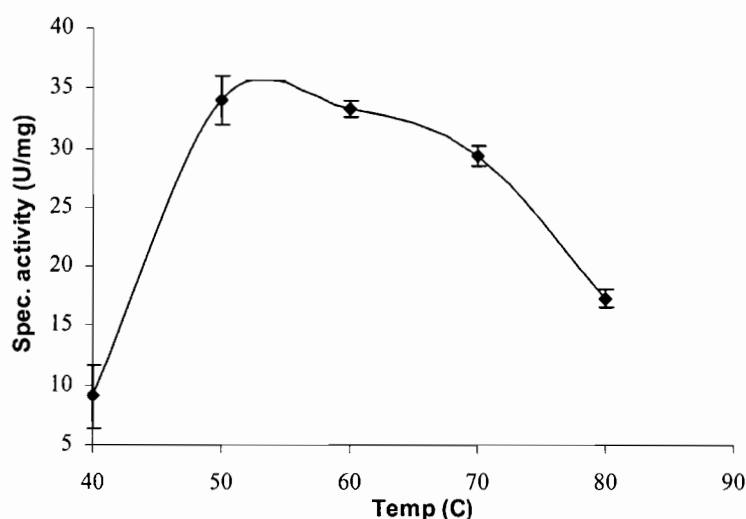


Figure 4.1: The effect of reaction temperature on immobilized amidase activity.

4.3.2.2 Determination of optimum working pH for the immobilized amidase

Reactions catalyzed by the immobilized amidase were conducted at pH ranging between 4.0 and 10.0. The specific activity of the amidase increased with increasing pH until a maximum at pH 7.0; above this pH, the specific activity decreased with increasing pH. Comparing with the results obtained for free amidase [section 3.3.2]; the specific activity of free amidase decreased rapidly above pH 7.0 until complete inactivation at pH 12.0 [Figure 3.2] while the specific activity of immobilized amidase decreased slowly above pH 7.0 and still maintained significant activity at pH 12.0 [Figure 4.2]. The results suggest that immobilization improved the pH stability of the amidase. Binding of the amidase amino acid groups to the oxirane groups of Eupergit C and cross-linking of enzyme molecules using glutaraldehyde and EDAC seemed to have protected the amidase active site from titration by basic pH.

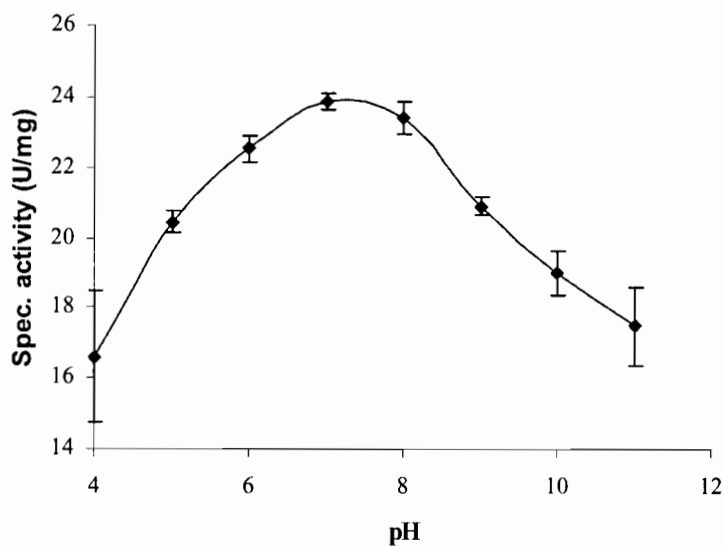


Figure 4.2: The effect of pH on immobilized amidase activity.

4.3.2.3. Determination of thermal stability of the immobilized amidase

The thermostability of the immobilized amidase was tested by incubating the biocatalyst at temperatures between 50°C and 80°C, for a period of 3 hours. The immobilized amidase showed high thermal stability at 50 and 60°C, maintaining more than half of its initial activity even after 3 hours of incubation [Figure 4.3]. The immobilized amidase only lost about 27% of its initial activity at both these temperatures, suggesting that it has a half-life of more than 3 hours at 50 and 60°C.

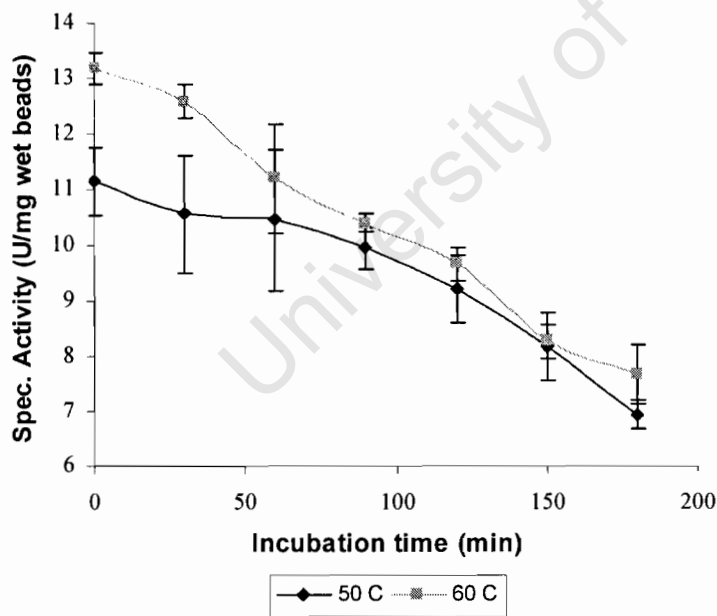


Figure 4.3: Activity of the immobilized amidase at 50 and 60°C.

In comparison to the free amidase thermostability [section 3.3.3], the immobilized amidase showed improved thermal stability. Although the immobilized amidase activity decreases rapidly with time at 70 and 80°C, the enzyme maintained significant activity until complete inactivation after about 150 minutes of incubation at 80°C. In comparison, the free amidase was completely inactivated in less than 60 minutes of incubation at 80°C [Figure 3.4]. These results agree with the earlier suggestion that immobilization of the amidase increased its thermal stability, giving it the ability to catalyze reactions even at higher temperatures.

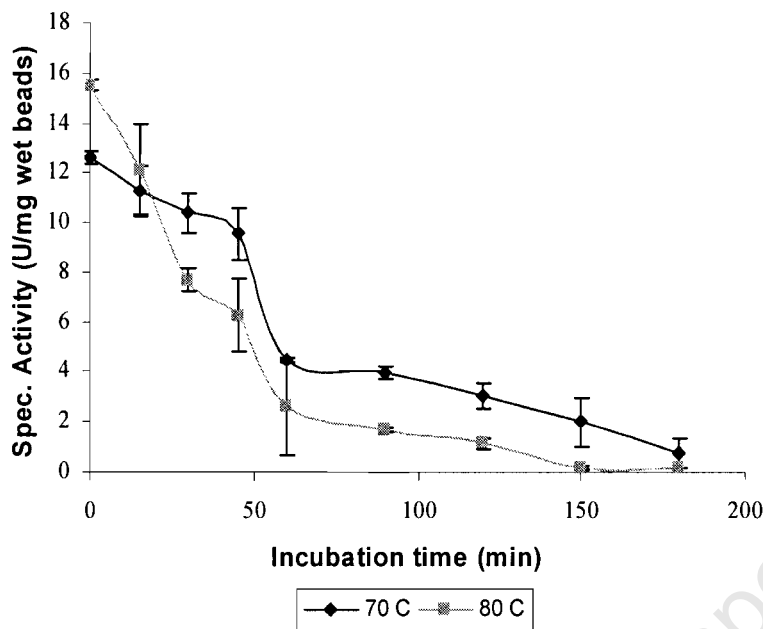


Figure 4.4: Variation of activity of the immobilized amidase at 70 and 80°C.

4.4. GENERAL DISCUSSION AND CONCLUSIONS

Many immobilization techniques have been described and employed for the stabilization of whole cells and isolated enzymes. A number of amidases have been successfully immobilized and utilized at industrial scale. Currently, no immobilization technique that has been specifically chosen the best method for stabilization of enzymes, but for industrial enzymes, covalent immobilization is usually the best choice [Torres-Bacete *et al.*, 2001]. As discussed in section 4.1.2, the form of the biocatalyst, whole cells or isolated enzyme, the physical and biochemical properties of the enzyme and the anticipated nature of the bioreactor process to be utilized are some of the factors that affect the choice of an immobilization technique. The cost of immobilization and the applicability of the technique on industrial scale also play a major role on the choice of a technique.

The different immobilization techniques used to stabilize the amidase from *Bacillus* sp. RAPc8 showed some interesting results. Some techniques resulted in both low protein binding yield and recovered amidase activity. Others resulted in high protein binding yield but low recovered amidase activity, while others resulted in high amidase activity but low protein binding yield [Table 4.1]. The best technique was selected as the method in which high protein binding yield and recovered amidase activity was obtained. However, it was noted that covalent attachment of the amidase on Eupergit C beads at 25°C resulted in low protein binding yield but maintained high amidase activity. Modification of this immobilization technique by subsequent cross-linking with glutaraldehyde and EDAC significantly improved the protein binding yield, although high concentrations of the cross-linking agents inhibited amidase activity. From the results shown in Table 4.1, it can be concluded that the combination of covalent attachment with subsequent cross-linking was the best immobilization technique in terms of maintaining both high protein binding yield and recovered amidase activity. It can also be concluded that cross-linking reagents (i.e. EDAC and glutaraldehyde) had inhibitory effects on the amidase at concentrations above 1.0w/v%. It is therefore recommended that the concentration of the cross-linking agent be optimized, in order to avoid inhibition of the enzyme. Similar and differing results have been reported in literature. Torres-Bacete *et al.* (2001) studied the thermal and pH stability of a penicillin V acylase isolated from a *Streptomyces lavendulae* strain, immobilized by covalent attachment on Eupergit C beads. They found that the half-life of the immobilized penicillin V acylase was 10-fold higher than that of the free/soluble enzyme at 40°C [Torres-Bacete *et al.*, 2001]. In the present study, the amidase from *Bacillus* sp. RAPc8 was immobilized using the same immobilization method. The half-lives of the free and immobilized amidase were found to be approximately 20 and 50 minutes at 70°C respectively. It was also found that at 80°C, the half-lives of free and immobilized amidase were approximately 15 and 30 minutes respectively. These results suggest that approximately 2.5- and 2.0-fold increases in thermal stability were achieved at 70 and 80°C respectively.

Enzyme immobilization has been reported to improve the stability of enzymes and sometimes alter the specificity of the enzyme altogether. Earlier in this study, it was reported that the optimum reaction temperature and pH of the amidase remained unchanged even after immobilization, but the immobilized enzyme was able to maintain significant activity at high and low temperatures and pH environment. Therefore, it can be concluded that although immobilization did not shift the optimal operating conditions of the amidase, its stability

across a wider range of both temperature and pH was improved. The same can be said for the thermostability of the amidase. The free amidase was completely inactivated in less than 60 minutes of incubation at 80°C, while the immobilized amidase was only completely inactive after 150 minutes of incubation at the same temperature. These findings suggest that immobilization improved the thermal stability of the amidase. Torres-Bacete *et al.* (2001) further studied the optimum operating conditions of both the free- and immobilized-penicillin V acylase in terms of reaction temperature and pH stability. Their results showed that the optimum working pH of the enzyme remained unchanged while the optimum reaction temperature increased by 10°C, from 50°C to 60°C. The latter finding differs from the results of this study, as both the reaction temperature and pH optima remained unchanged after immobilization.

In the case where immobilization affects the physical and biochemical properties of enzymes in a negative manner, reduced catalytic activity and stereoselectivity, and diffusion limitations are often cited. In the work reported in this chapter, such negative effects were observed in the low catalytic activity of the amidase immobilized in a polyacrylamide gel (section 4.3.1). Studies by Graham *et al.* (2000) on nitrile biotransformation using free and immobilized cells of the *Bacillus* sp. RAPc8 strain in alginate beads, has shown that the thermal and biochemical properties of the cells remained unchanged even after immobilization [Section 4.3.2]. Furthermore, their studies on initial rates showed that there were mass transfer limitations across the alginate barrier and possibly oxygen limitations to the cells. Conversely, whole cells of *Bacillus pallidus* immobilized in calcium alginate beads were more resistant to high concentrations of 3-cyanopyridine and nicotinic acid than the free cells [Mylerová and Martínlovà, 2003]. More interestingly, cells of *Candida guilliermondii* immobilized in alginate beads were able to hydrolyze substrates that were not converted by the free cells [Mylerová and Martínlovà, 2003].

The overall conclusion is that, since the choice of an immobilization technique is dependent on both the properties of the biocatalyst and that of the carrier, in order to choose a suitable immobilization technique, the physical (e.g. crystal and active site structure, and amino acid sequence) and biochemical (e.g. catalytic mechanism) properties of a biocatalyst must be fully understood. Knowledge of the physical and biochemical properties of an enzyme lead to the choice of a suitable carrier for immobilization, and application.

Chapter 5 Selection and production of a target product

5.1. INTRODUCTION

In chapter 3, the substrate specificity of the amidase from *Bacillus* sp. RAPc8 was discussed, and it was reported that enzyme is only capable of hydrolysing short- and mid-length aliphatic amides to their corresponding carboxylic acids and ammonia. In the work reported in this chapter, lactic acid was identified as a target product for potential commercial production. Lactic acid was first discovered by a Swedish chemist, Scheele, in 1780 and it was first produced commercially by Charles E. Avery at Littleton, USA in 1881. It is the most widely occurring carboxylic acid in nature [Narayanan *et al.*, 2004], and it can be manufactured by either chemical synthesis or carbohydrate fermentation. To date, approximately 50% of lactic acid has been produced by microbial fermentation, while the remainder is manufactured by chemical synthesis [VickRoy, 1985]. Lactic acid has various applications in the food, pharmaceutical, leather and textile industries [VickRoy, 1985], and since it contains both hydroxyl (-OH) and carboxylic groups (-COOH), its high reactivity makes it a good chemical feedstock for conversion into various chemicals including acrylic acid, propylene glycol, acetaldehyde and 2,3-pentanedione [Oh *et al.*, 2005]. In recent years, the demand for lactic acid has increased due to its application in the preparation of biodegradable polymers, medical sutures, and green solvents [Oh *et al.*, 2005].

Due to the substrate specificity and chiral selectivity of the amidase characterized in this study, and the growing demand for lactic acid, a new method for the commercial production of lactic acid is proposed. The advantages and disadvantages of current lactic acid production methods (chemical and fermentation) have been considered, and theoretically, the newly proposed method could be competitive in terms of cost, optical purity, nutrient requirements and by-product formation. The proposed method involves the enzymatic hydrolysis of lactonitrile to produce lactic acid and ammonia, using a nitrile hydratase-amidase continuous process. In the work reported in this chapter, the uses and industrial applications of lactic acid have been discussed, and also, theoretical comparisons have been made between the current industrial methods for lactic acid production and the newly proposed method.

5.1.1. Industrial applications of lactic acid

Food-related applications are a major use of D,L-lactic acid in the United States, and these account for 85% of the commercial product [Garlotta, 2002]. D,L-lactic acid is used as an acidulant, pH-buffering agent, flavouring agent, and bacterial inhibitor in a wide variety of processed foods [Narayanan *et al.*, 2004]. Addition of aqueous lactic acid solution to the packaging of poultry and fish increases their shelf life [Anon, 1992]. Esters of lactic acid are used as emulsifying agents in baking foods [Narayanan *et al.*, 2004]. D,L-lactic acid is also used in small scale industrial applications such as pH adjustment hardening baths for cellophanes used in food packaging, as the terminating agent for phenol formaldehyde resins, alkyd resin modifier, solder flux, in lithographic and textile printing developing, adhesive formulations, electroplating and electropolishing baths, and as detergent builder. In the cosmetic industry, lactic acid is used in topical ointments, lotions, anti acne solutions, humectants, parenteral solutions and dialysis applications [Narayanan *et al.*, 2004].

In the medical field, lactic acid has increasing applications in the preparation of biodegradable polymers, medical sutures, and green solvents [Oh *et al.*, 2005]. Present research in the medical field focuses on the application of biopolymers such as polylactic acid (PLA), polyglycolic acid (PGA), chitin, chitosan, hyaluronan, and their derivatives. PLA and related poly (α -hydroxy) acids are widely used as biological scaffolds [Jain, 2000 and Rokkanen *et al.*, 2000] and bio-absorbable implants [Yao *et al.*, 2004] in the medical field. PLA is also one of the few polymers in which the 3-dimensional structure can easily be modified by polymerizing a desired mixture of the L- and D-isomers to yield high molecular-weight amorphous polymers that can be used for food purposes [Garlotta, 2002]. Lactic acid and its derivatives are the monomers used for ring-opening polymerization, to produce biodegradable PLA polymers. The presence of optically pure L-lactic acid gives PLAs with high melting points and high crystallinity [Lunt, 1998; Yun and Ryu, 2001]. Devices made of PLA-PGA copolymers have been used for the controlled release of antibiotics, anticancer and antimalarial agents, contraceptives, hormones, insulin, narcotic antagonists, and proteins [Nurhan, 2003].

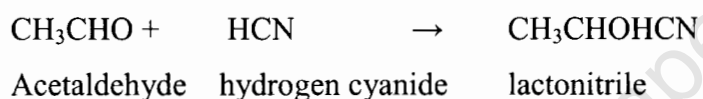
Ongoing research on the application of PLA-PGA copolymers focuses on their use as sutures, clips, staples, and reinforcement materials. These polymers are widely used as sutures in surgery because they degrade within the body after the incision has healed [Nurhan, 2003].

The world market for lactic acid is growing every year [Wasewar *et al.*, 2004]. Approximately 350 000 tonnes of lactic acid is produced annually, and the worldwide growth is believed to be 12-15% per year [Wasewar *et al.*, 2004].

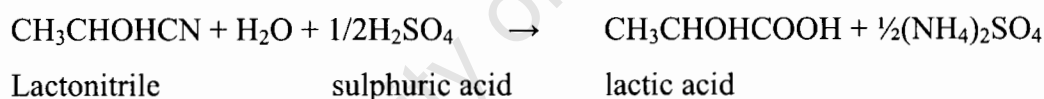
5.1.2. Chemical synthesis of lactic acid

The commercial chemical synthesis of lactic acid involves the use of lactonitrile, which is produced by the reaction of hydrogen cyanide with acetaldehyde in the presence of a base (calcium hydroxide). This liquid phase reaction occurs at high atmospheric pressures, and crude lactonitrile is recovered and purified by distillation [Narayanan *et al.*, 2004]. Lactic acid is formed by the acid hydrolysis of lactonitrile using either concentrated HCl or H₂SO₄, which produces the corresponding ammonium salt and lactic acid. The recovery of lactic acid is achieved by esterification with methanol to produce methyl lactate, which is separated and then purified by distillation, and hydrolysis by water with an acid catalyst to produce lactic acid and the methanol. The chemical synthesis process can be illustrated by the following reactions:

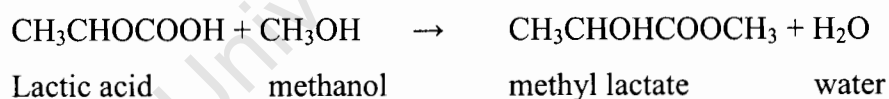
i. Lactonitrile production:



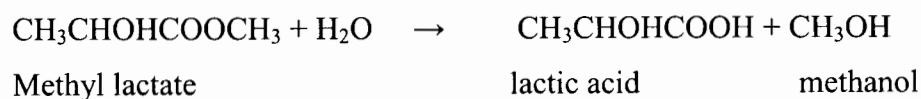
ii. Acid hydrolysis:



iii. Esterification:



iv. Hydrolysis:



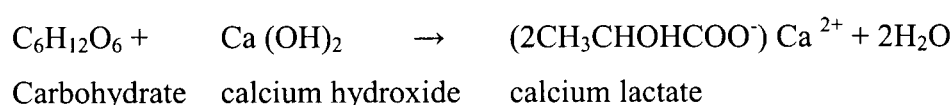
5.1.3. Carbohydrate fermentation

Lactic acid production by fermentation involves the use of lactic acid bacteria. The choice of an organism depends primarily on the carbohydrate to be fermented [Narayanan *et al.*, 2004]. *Lactobacillus delbreuckii* subspecies *delbreuckii* is able to ferment sucrose; *Lactobacillus helveticus* is able to ferment both lactose and galactose; *Lactobacillus amylophylus* and *Lactobacillus amylovorus* are able to ferment starch; and *Lactobacillus lactis* can ferment glucose, sucrose and galactose [Narayanan *et al.*, 2004].

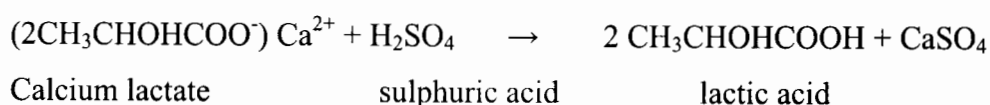
Lactobacillus species, in general, have complex nutritional requirements, as they are unable to synthesize their own growth factors. Their growth media must contain, among other compounds, a carbon source, a nitrogen source and other nutrients such as B-vitamins, amino acids and nucleotides. These nutrients are usually supplied by corn steep liquor, yeast extract, cottonseed flour, or soy flour [Garlotta, 2002].

Commercial fermentation is usually carried out in a batch process and takes three to six days to complete [Garlotta, 2002]. The efficiency of fermentation is determined by lactic acid yield in the final broth, but high concentrations of lactic acid lead to growth inhibition and toxicity [Garlotta, 2002]. Several methods for neutralizing and extracting lactic acid have been developed in an attempt to maintain high efficiency and cell growth. Addition of calcium hydroxide or calcium carbonate to neutralize the acid and give soluble calcium lactate solution is one example. The calcium lactate broth is filtered to remove cells and other insolubles, then evaporated, recrystallised, and acidified with sulphuric acid, to yield lactic acid and calcium sulphate [Garlotta, 2002]. The insoluble calcium sulphate is removed by filtration and discarded. Lactic acid is recovered by hydrolysis with sulphuric acid, esterification with methanol, distillation and hydrolysis with water in the presence of an acid catalyst [Narayanan *et al.*, 2004]. The carbohydrate fermentation process can be illustrated by the following reactions:

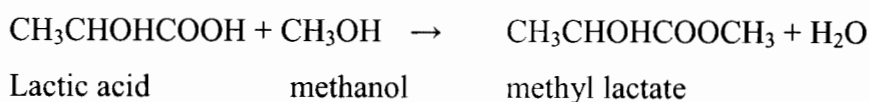
i. Fermentation and neutralization:



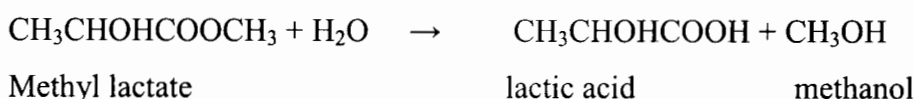
ii. Acid hydrolysis:



iii. Esterification:



iv. Hydrolysis:

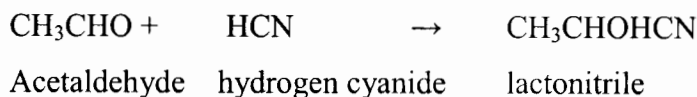
**5.1.4. Nitrile biotransformation**

The method proposed here involves the use of an immobilized two-enzyme system. A nitrile hydratase can be used to hydrolyze lactonitrile to lactamide, followed by hydrolysis, using an amidase, of the lactamide to produce lactic acid, and ammonia as the only by-product. In this study, the biochemical characteristics of the amidase from *Bacillus* sp. RAPc8 have been studied and reported, while in another study in our laboratory, an industrial biocatalyst is being developed from a nitrile hydratase isolated from the same *Bacillus* sp. RAPc8 strain. Preliminary studies of the nitrile hydratase have shown that the enzyme is capable of hydrolyzing short- and mid-length aliphatic nitriles, and cyanopyridine to their corresponding amides [Cameron, PhD thesis, 2001], while the biochemical properties of the amidase were reported in previous chapters of this study.

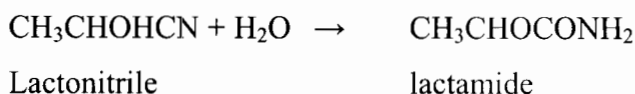
The proposed method involves a two-stage hydrolysis of lactonitrile to produce lactic acid and ammonia, with the recovery and separation of ammonia either by distillation or stripping. Lactonitrile can be synthesized by the reaction of acetaldehyde with hydrogen cyanide in the presence of a base [Narayanan *et al.*, 2004]. The two biocatalysts, a nitrile hydratase and the amidase, could be immobilized or co-immobilized in a suitable carrier (Eupergit CM beads in the case of the amidase) and then used in a dual-enzyme reactor process for a two-stage continuous production of lactic acid. Ammonia, the only by-product, is relatively volatile while lactic acid is not, and therefore distillation could be used for its separation. Other

simpler procedures for the removal of ammonia in solution include stripping. The nitrile biotransformation process can be illustrated by the following reactions:

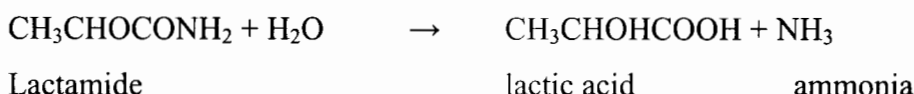
i. Lactonitrile production:



ii. Nitrile hydrolysis:



iii. Amide hydrolysis:



5.1.5. Rationalization of the nitrile biotransformation method

The main advantages of the nitrile biotransformation method are that, potentially, the immobilized biocatalysts can be recycled and re-used, the process can be continuous, high productivity can be achieved in just few hours, and ammonia is the only final by-product. These advantages are in contrast to the current fermentation method, discussed earlier in this chapter [section 5.1.3], in that the process is conducted in a batch process.

Both the fermentation and nitrile biotransformation methods have the advantage that optically pure lactic acid can be obtained by choosing an enantioselective biocatalyst or microorganism, whereas the chemical synthesis results in racemic mixtures of lactic acid [Oh *et al.*, 2005]. One of the main disadvantages of both the chemical and fermentation methods is the generation of large volumes of salt wastes, which can pose a serious disposal and management problem under industrial-scale processes [www.es.anl.gov/htmls/food.process.html], whereas nitrile biotransformation only generates ammonia as a by-product, which can be separated and sold for agricultural applications (fertilizer production). The buffer solution (i.e. phosphate), which is used for dissolving the substrate and buffering of the biocatalyst in the nitrile biotransformation method, could be separated by precipitation or dialysis.

In sections 5.1.2 and 5.1.3, it was shown that the recovery of lactic acid involves precipitation of calcium lactate using calcium hydroxide. The calcium lactate is recovered by filtration, and converted to lactic acid by the addition of sulphuric acid. Also, the dilute lactic acid product is then sequentially purified using activated carbon, evaporation, and crystallization [Wasewar *et al.*, 2004]. According to Wasewar *et al.* (2004), these separation and purification steps account for up to 50% of the production costs, due to the expensive consumption of lime and sulphuric acid. Theoretically, separation and purification steps in the nitrile biotransformation method will involve ammonia stripping, phosphate buffer precipitation and concentration of the lactic acid by evaporation of water, and these steps will account for less than 50% of the production costs.

Lactic acid for use in biological polymers, pharmaceuticals, and/or food derivatives requires further purification to remove by-product proteins and carbohydrates from the fermentation broth. Higher purity is usually obtained by distillation of the acid as methyl or ethyl ester, followed by hydrolysis back to the acid [Garlotta, 2002]. This requirement also increases the production costs due to the additional separation unit and further increase in the amount by-product.

The scope of this study was limited to the study of the amidase step in the production of lactic acid. The work reported here covers the production of lactic acid using the immobilized amidase in a continuous fixed-bed reactor.

5.2. MATERIALS AND METHODS

5.2.1. Materials

All chemicals were purchased from Sigma-Aldrich (SA), Aldrich-SA and Fluka except for D-lactamide and L-lactamide, which were purchased from Sigma-Aldrich (International). All solvents were purchased from Merck-SA. Eupergit CM was received as a gift from Röhm Pharma Polymers (Darmstadt, Germany) and the biocatalyst was prepared as described in Chapter 4.

5.2.2. Measurement of reaction rates for D-lactic acid production

The reactions were carried out in glass reaction tubes and final D-lactamide concentrations of between 10 and 100mM were used. 200mg of immobilized amidase was mixed with 3.0ml of phosphate buffer, pH 7.0, and then pre-incubated at 50°C for 5 minutes. A required amount of aqueous D-lactamide was added immediately and the mixture was incubated at 50°C for 60 minutes. 100µl samples were withdrawn after 3, 5, 7, 10, 20, 30, 45 and 60 minutes of incubation, and analyzed for amidase activity using methods described in chapter 2.

5.2.3. Determination of initial rate kinetics

Similar to the reaction rate experiments described in section 5.2.2, initial rate kinetics reactions were carried out in glass reaction tubes, and final substrate concentrations of between 10 and 60mM were used. 200mg of immobilized amidase was mixed with 3.0ml of phosphate buffer, pH 7.0, and then pre-incubated at 50°C for 5 minutes. A required amount of aqueous D-lactamide was added and the mixture was incubated at 50°C for 20 minutes. 100µl samples were taken after 4, 8, 12, 16 and 20 minutes of incubation and analyzed for amidase activity.

5.2.4. Investigation of potential biocatalyst inhibition

5.2.4.1. Substrate inhibition by D,L- and L-lactamide

D,L- and L-lactamide concentrations of between 10 and 100mM were used. In glass reaction tubes, 100µl of the amidase solution (2.0mg/ml) mixed with 3.0ml of phosphate buffer, pH 7.0, was pre-incubated at 50°C for 5 minutes. A required amount of the lactamide was added and the reaction mixture was incubated for 60 minutes at 50°C. 100µl samples were withdrawn after 60 minutes of incubation, and used to determine the product concentration using the ammonia detection assay described in chapter 2.

5.2.4.2. Product inhibition by D,L-lactic acid

All the reactions were carried out in a glass reaction tube and D,L-lactic acid concentrations of between 0 and 100mM were used. A mixture of buffer, 100µl purified amidase extract and required amount of the D,L-lactic acid was pre-incubated for 5 minutes at 50°C. D-lactamide was added to a final concentration of 40mM and the reaction mixture was incubated for 30 minutes at 50°C. 100µl samples were removed at the end of the reaction and were used to determine the product concentration using the ammonia detection assay.

5.2.4.3. Product inhibition by D,L-lactic acid, with pH control

The reactions were carried out as outlined in section 5.2.4.2 with the modification of adjusting the pH to 7.0, after addition of the acid, using a 1M NaOH solution. A mixture of phosphate buffer (pH 7.0) and a required amount of the D,L-lactic acid was adjusted back to pH 7.0 prior to addition of enzyme. 100µl of purified amidase extract (2.0 mg/ml) was then added and the mixture was pre-incubated for 5 minutes at 50°C. The reactions were then carried out as described in section 5.2.4.2.

5.2.5. Optimisation of reaction conditions for lactic acid production

The parameters used to optimize the productivity of lactic acid were reaction time and enzyme concentration. The first experiment involved optimization of reaction time for maximum production of lactic acid. In glass reaction tubes, a mixture of phosphate buffer, pH 7.0, and amidase biocatalyst amounts of between 50 and 500mg were pre-incubated for 5 minutes at 50°C. D-lactamide was then added to a final concentration of 40mM and the reaction mixture was incubated for 3 hours at 50°C. 100µl samples were withdrawn every 30 minutes and used to determine the product concentration using the ammonia detection assay.

The second experiment involved the determination of optimum enzyme concentration for maximum production of lactic acid. In glass reaction tubes, a mixture of phosphate buffer, pH 7.0, and 40mM lactamide was pre-incubated for 5 minutes at 50°C. A required amount of the amidase biocatalyst (between 50 and 500mg) was then added and the mixture was incubated for 3 hours at 50°C. 100µl samples were removed every 30 minutes and the product concentration was determined as described previously.

5.3. RESULTS

5.3.1. Measurement of reaction rates for D-lactic acid production

Reaction rates for D-lactic acid formation, catalyzed by the amidase biocatalyst, using D-lactamide as a substrate, were determined over 60 minute periods using D-lactamide concentrations of between 10 and 100mM. The concentration of the ammonia product, which is equivalent to the concentration of the D-lactic acid formed, was measured using methods described in section 2.2.7. The reactions were found to follow Michaelis-Menten type kinetics, in which product formation (concentration) should increase exponentially with time until stationary-phase, where the reaction reaches completion. Figure 5.1 shows that at each D-lactamide concentration, the product concentration increased exponentially until the reactions had reached completion (i.e. curves become horizontal), observed by the constant product concentration over the time period. For D-lactamide concentrations between 10 and 60mM, the exponential increase in product concentration was observed between 0 and 30 minutes of the reaction, whereas for concentrations above 60mM, the increase was observed between 0 and 10 minutes of the reaction [Figure 5.1]. Therefore, unexpectedly, the reactions at higher D-lactamide concentrations (above 60mM) stopped earlier than expected. This result could be due to biocatalyst inhibition by either high substrate or product concentrations. The reaction rate curves, shown in Figure 5.1, were used to determine the reaction time range in which to measure Michaelis-Menten kinetic constants, the optimum substrate concentration and the optimum reaction period for maximum conversion of the substrate. It can be observed that the highest rate of reaction was achieved with a substrate concentration of 40mM, and that the reaction reached completion within 40 minutes of incubation.

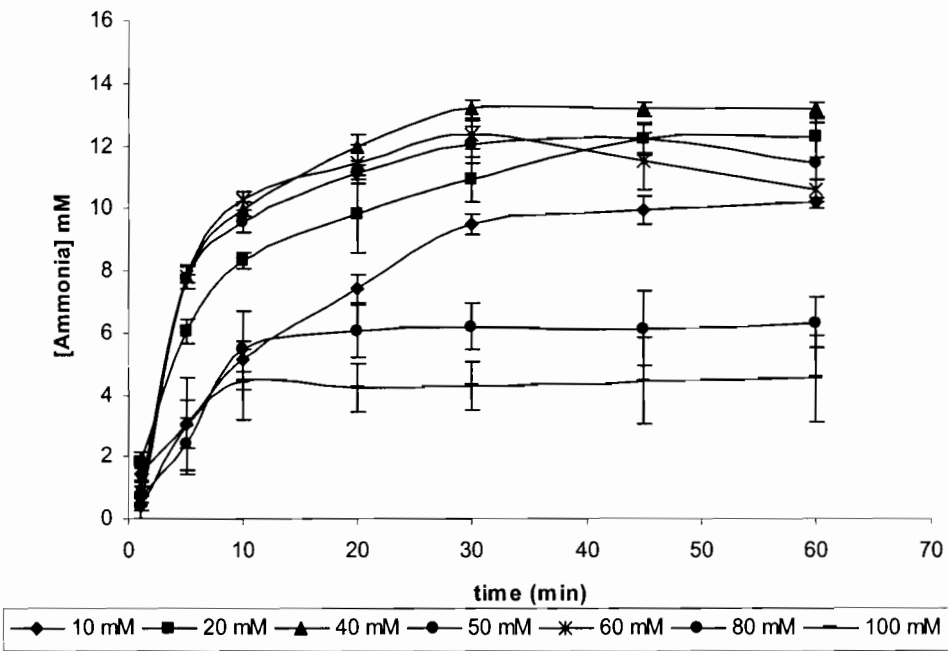


Figure 5.1: Reaction rates of the amidase with D-lactamide as a substrate at different concentrations, using 0.55mg/ml amidase stock solution.

5.3.2. Determination of initial rate kinetics and kinetic constants

Initial rate kinetic constants were determined by performing reactions similar to those described in section 5.3.1, but over 20 minute periods (i.e. exponential phase of the reactions). Based on the fact that the reaction rates decreased above 60mM D-lactamide concentration [section 5.3.1], the reactions were performed at D-lactamide concentrations between 10 and 60mM. As expected, linear plots were obtained over the 20 minute reaction periods [Figure 5.2]. The linear plots were used to determine the rate of reaction at each substrate concentration, defined as the slope of straight line obtained from the plot of product concentration versus time. Table 5.1 summarises the linear equations and the R^2 values estimated from the linear plots shown in figure 5.2.

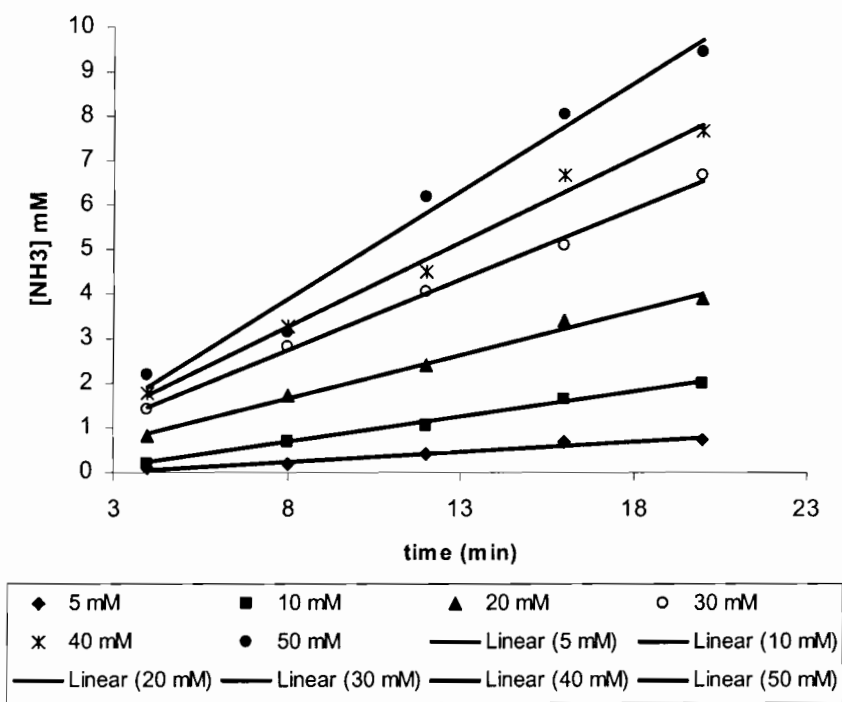


Figure 5.2: The plot of product concentration versus time during the exponential phase of the reaction at different D-lactamide concentrations, conducted in 3.0ml, using 0.55mg/ml amidase stock solution.

Table 5.1: Summary of the linear equations obtained from the initial rate plots.

Substrate concentration (Mm)	Initial rate equation	R ² value
10	y = 0.0349x + 0.1132	0.9720
20	y = 0.075x + 0.01195	0.9963
30	y = 0.1289x + 0.3504	0.9913
40	y = 0.2103x + 0.4014	0.9966
50	y = 0.2507x + 0.4326	0.9894
60	y = 0.3215x + 0.2485	0.9765

5.3.2.1. Michaelis-Menten kinetic constants

For enzyme reactions that follow the Michaelis-Menten type kinetics, the rate of substrate utilization can be defined by the following equation:

$$r_s = \frac{V_{max} \cdot [S]}{K_m + [S]}$$
equation 5-1

where V_{\max} = the maximum rate of reaction for a given enzyme concentration
 K_m = Michaelis constant
 $[S]$ = Substrate concentration
 r_s = rate of reaction

The kinetic constants were determined by linearising the Michaelis-Menten equation to obtain the following equation:

$$\frac{1}{r_s} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \dots\dots\dots\text{equation 5-2}$$

Equation 5-2 is known as the Lineweaver-Burk plot and was used here to determine the kinetic constants K_m and V_{\max} . The y-intercept of the Lineweaver-Burk plot was used to determine the value of V_{\max} , while the slope of the line was used to determine the value of K_m .

The catalytic efficiency of enzyme was determined from the Michaelis-Menten constants and it was defined by the following equation:

$$k_{\text{cat}} = \frac{V_{\max}}{E_o} \dots\dots\dots\text{equation 5-3}$$

where E_o = initial enzyme concentration

Figure 5.3 shows the Lineweaver-Burk plot obtained using an immobilized amidase and D-lactamide as a substrate, and Table 5.2 summarises the estimated values of the kinetic constants.

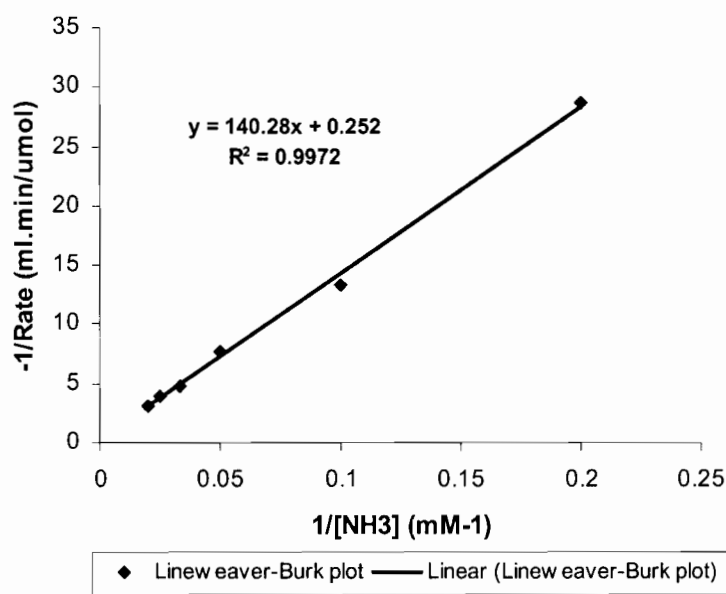


Figure 5.3: The Lineweaver-Burk plot to determine enzyme kinetic parameters of amidase-catalyzed D-lactic acid production.

Table 5.2: The estimated values of the Michaelis-Menten kinetic parameters based on the slope and y-intercept from figure 5.3.

Kinetic constants	Value
K_m ($\mu\text{mol/ml}$)	556.7
V_{max} ($\mu\text{mol/ml.min}$)	3.97
k_{cat} (sec^{-1})	0.43

5.3.3. Investigation of biocatalyst substrate inhibition

In chapter 3, the enantioselectivity of the amidase was investigated and it was found to be D-specific towards lactamide. Potentially, both the D- and L-isomers could inhibit the amidase biocatalyst at high concentrations. In the work reported in this section, the potential inhibition of the amidase biocatalyst, by D,L-lactamide or L-lactamide, at different concentrations, was investigated.

5.3.3.1. Inhibition by the substrate D,L-lactamide

Amidase-catalyzed reactions were carried out with D,L-lactamide as both the substrate and potential inhibitor, at varying concentrations. The specific activity of the amidase, based on product formation, increased with increasing D,L-lactamide concentration up to a maximum at 40mM of the substrate. Above a concentration of 40mM D,L-lactamide, the specific activity decreased with increasing D,L-lactamide concentration. The presence of L-lactamide in the reaction mixture, which the amidase is unable to hydrolyze, could, therefore be the cause of inhibition. Figure 5.3 shows the amidase activity profile at different substrate (D,L-lactamide) concentrations.

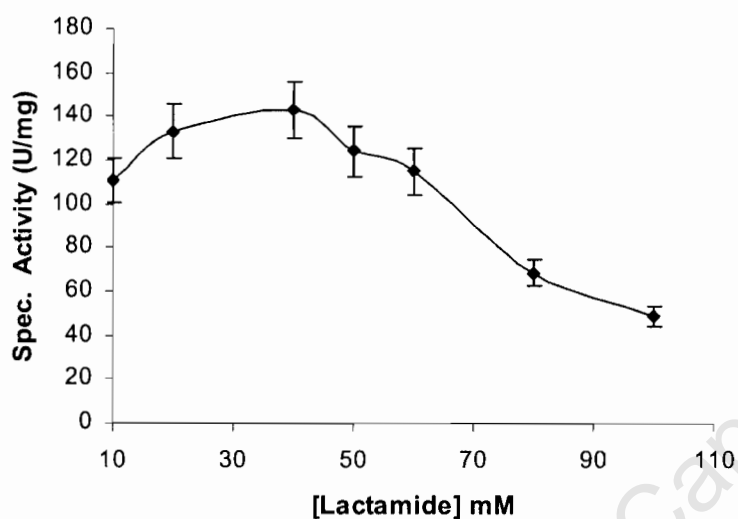


Figure 5.3: Inhibitory effects of D,L-lactamide on the amidase activity measured over a range of concentrations.

5.3.3.2. Inhibition by the L-lactamide

Amidase-catalyzed reactions were carried out with D-lactamide as a substrate, and varying concentrations of L-lactamide were added, since it was considered to be a potential inhibitor. Figure 5.4 shows the inhibition profile of the amidase by L-lactamide. The specific activity of the amidase, based on product formation, decreased exponentially with increasing L-lactamide concentration. The results suggest that L-lactamide has an inhibitory effect on the amidase, since the amidase is unable to hydrolyze it.

The pH measurement of the reactions shows that the pH remained between 7.0 and 8.0, the range in which the amidase exhibits maximum activity, and therefore pH effects were not expected to contribute to this inhibition effect [Figure 5.4]. The change in pH from 7.0 at low L-lactamide concentrations (<50mM) to 8.0 at high L-lactamide concentrations (>50mM) could be attributed to the basic nature of amides [Figure 5.4]. Therefore, L-lactamide concentrations below 50mM had no effect on the pH of the reaction, while L-lactamide concentrations above 50mM decreased the pH of the reaction to approximately 8.0.

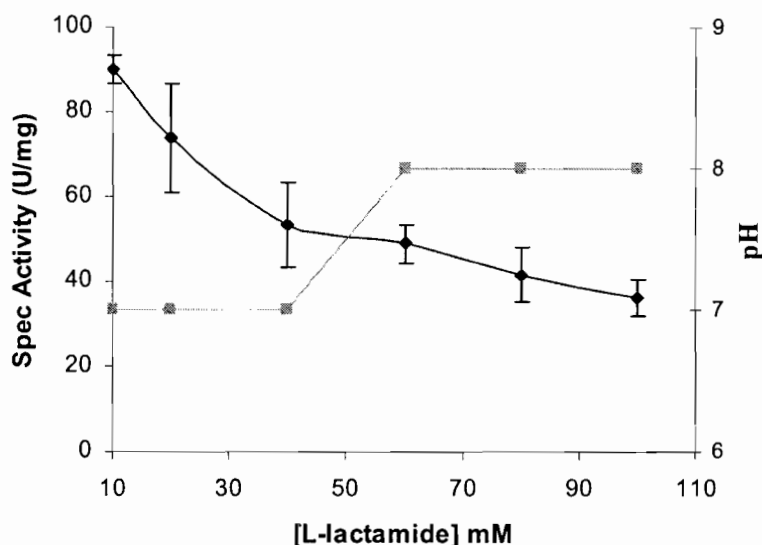


Figure 5.4: Inhibitory effects of L-lactamide on the amidase activity measured over a range of concentrations, using D-lactamide as a substrate.

5.3.4. Investigation of biocatalyst product inhibition

Lactic acid could potentially cause inhibitory effects on the amidase in two ways. Firstly, it could be a potential competitive inhibitor of the amidase due to the similarity of its chemical structure to that of the substrate (lactamide). Secondly, it could cause inhibitory effects by lowering the pH of the reaction medium, and thus causing an acid titration of the enzyme. In the work reported in this section, both potential inhibition effects by lactic acid were investigated. The experiments were limited to the use of the racemic D,L-lactic acid as a potential product inhibitor, due to availability.

5.3.4.1. Inhibition by the product D,L-lactic acid

Amidase-catalyzed reactions using D-lactamide as a substrate, in the presence of varying concentrations of D,L-lactic acid, were carried out according to the method described in section 5.2.4.2. The inhibition profile shown in figure 5.5 suggests that D,L-lactic acid is a strong inhibitor of the amidase enzyme. The specific activity of the amidase first increased to a maximum (~200 U/mg), at a concentration of 20mM D,L-lactic acid. Above 20mM D,L-lactic acid concentration, the specific activity decreased until complete inhibition at and above 60mM of the acid. The pH profile measurement of the reactions [Figure 5.5] shows that the pH of the reaction mixture decreased linearly with increasing D,L-lactic acid concentration. Therefore, acid titration of the enzyme at low pH values (less than 7.0) could be the cause of amidase inhibition by D,L-lactic acid, rather than competitive inhibition.

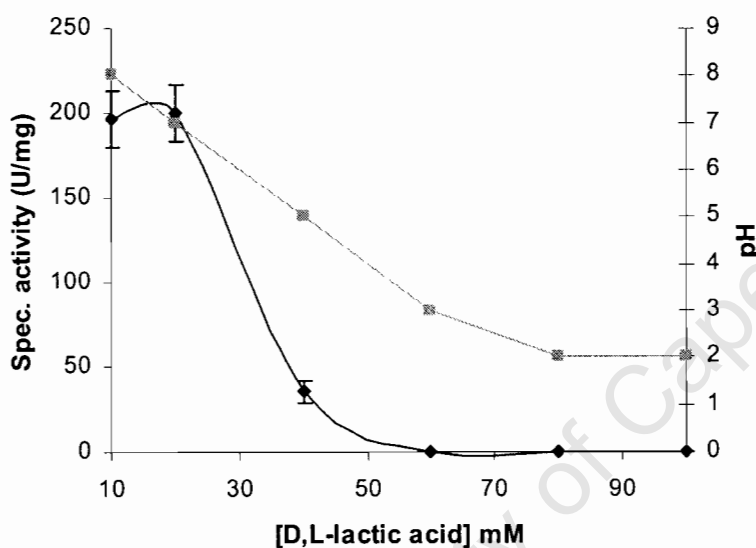


Figure 5.5: Inhibitory effects of D,L-lactic acid on the amidase activity measured over a range of concentrations.

5.3.4.2. Inhibition by D,L-lactic acid with pH adjustment

Amidase-catalyzed reactions were carried out as described in section 5.3.4.1, with the difference being the adjustment of the pH to 7.0 prior to addition of the enzyme. In section 5.3.4.1, it was suggested that increasing concentrations of D,L-lactic acid lowered the pH of the reaction mixture, thereby deactivating the enzyme. Figure 5.6 shows the activity profile of

the amidase, with increasing D,L-lactic acid concentrations. The specific activity of the amidase remained constant at the different D,L-lactic acid concentrations, when the pH was controlled at pH 7.0. The results suggest that acid titration is the main form of inhibition of the amidase by D,L-lactic acid.

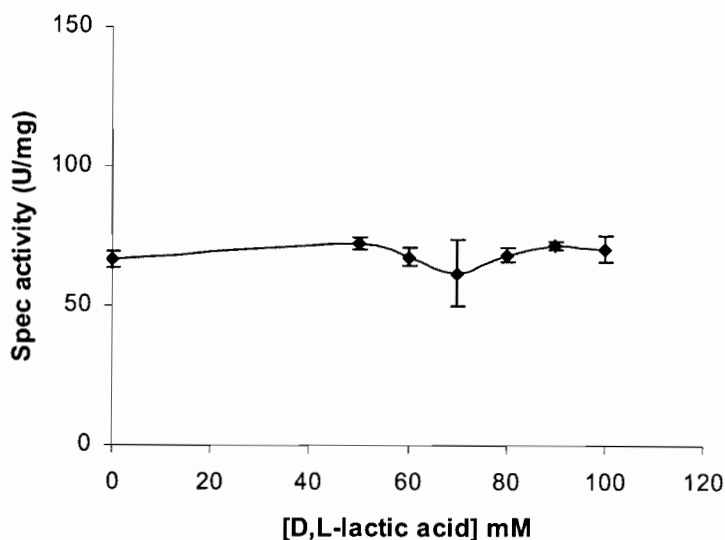


Figure 5.6: Inhibitory effects of D,L-lactic acid on the amidase activity measured over a range of concentrations, with pH adjustment.

5.3.5. Optimisation of D-lactic acid production based on enzyme loading and reaction time

The yield of a desired product can be optimised using a number of operating parameters, including temperature, pressure, pH, substrate and product concentration, amount of biocatalyst and the reaction/residence time. In the work reported in this section, the relationship between the yield of lactic acid, defined by conversion as $\frac{[So]-[S]}{[So]}$, the amount of biocatalyst and the reaction time were investigated.

5.3.5.1. The effect of enzyme loading on the production of D-lactic acid

Amidase-catalyzed reactions were carried out with varying amounts of immobilized amidase biocatalyst. The amount of D-lactamide converted to D-lactic acid was defined as the productivity (conversion) of the reaction. The conversion was found to increase with

increasing proportions of biocatalyst, until it reached a plateau at and above 400mg of immobilized beads. The highest conversion of substrate to product was estimated to be 60% [Figure 5.7]. The inability of the reactions to reach 100% conversion could be attributed to a possible mass transfer limitation, and a short reaction period for achieving 100% conversion.

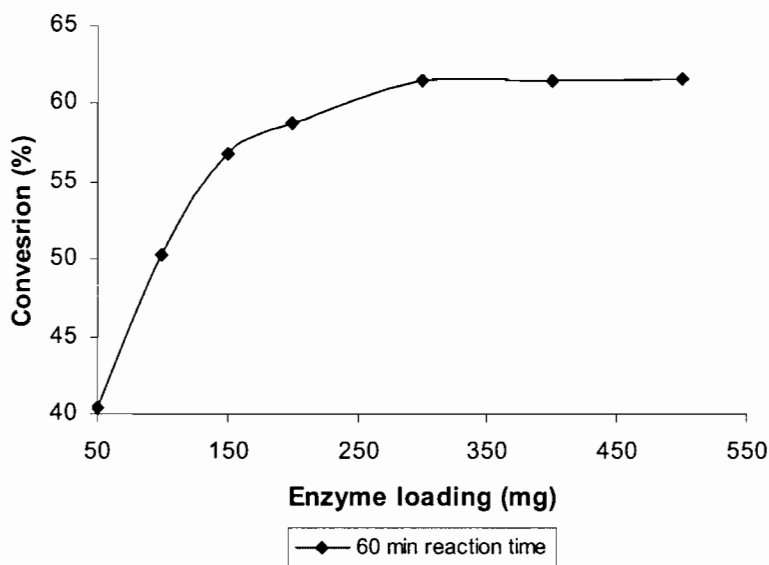


Figure 5.7: The effect of enzyme loading measured as the amount of biocatalyst (mg) in the reaction mixture, on the conversion of D-lactamide to D-lactic acid.

5.3.5.2. The effect of reaction time on the production of D-lactic acid by the amidase biocatalyst

The amidase-catalyzed reactions were carried out using different amounts of biocatalyst, over a period of time. Over a reaction period of 150 minutes, the conversion of D-lactamide was found to increase more- or –less linearly with time, for almost all the enzyme concentrations. A linear increase suggests that, at that particular biocatalyst loading/concentration, the reaction was still on the exponential phase. The biocatalyst loading between 300 and 500mg shows that stationary phase (plateau) was reached after approximately 60 minutes of the reaction, and maximum conversion achieved was approximately 60%. Therefore, the 150 minutes reaction time was not sufficient to achieve 100% conversion of the substrate, under the experimental conditions.

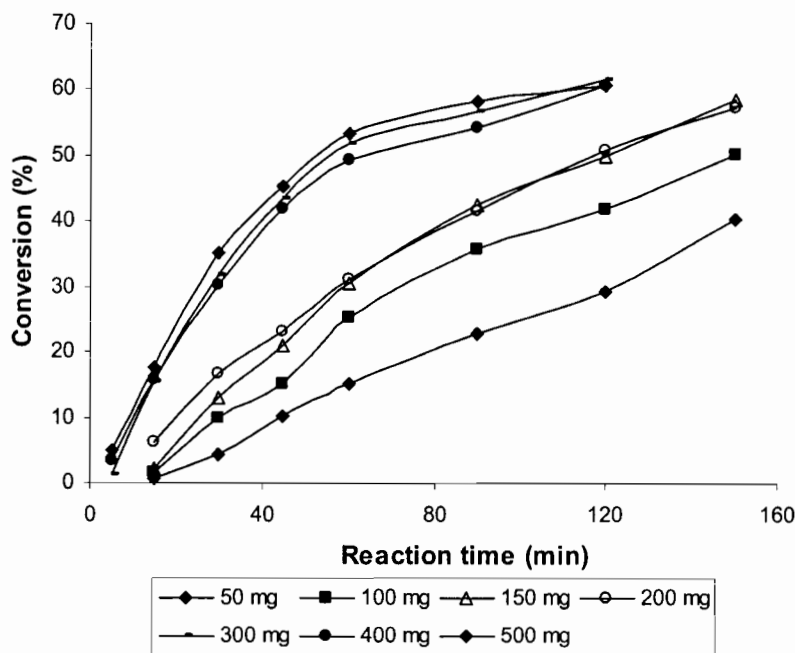


Figure 5.8: The effect of reaction time on the conversion of D-lactamide to D-lactic acid, at various biocatalyst concentrations (mg) in the reaction mixture.

5.9. GENERAL DISCUSSION AND CONCLUSIONS

The advantages and disadvantages of the current lactic acid production methods suggest that fermentation is currently the preferred method [section 5.1.2 – 5.1.3]. Furthermore, although the current commercial lactic acid production methods have a number of disadvantages associated with their operation, these methods have been used for the past few decades. Therefore, this illustrates a stable market and the growing demand for lactic acid, in the sense that even at high production costs, which often lead to high product price, chemical synthesis and carbohydrate fermentation, are still used effectively for the production of the acid. The recent application of lactic acid derived polymers (PLA) in the preparation of biodegradable polymers and medical sutures is one of the main reasons for the increase in demand for lactic acid. In theory, the method proposed here (nitrile biotransformation) has more advantages than the current commercial fermentation method [section 5.1.4] and therefore it was suggested that the nitrile biotransformation method might replace current lactic acid production methods. The main disadvantage in the use of the amidase biocatalyst (biotransformation method) for the production of lactic acid was biocatalyst inhibition by both substrate and product [section 5.3.3]. Potential inhibition of the amidase by substrate and/or

product can take the form of competitive-inhibition and/or deactivation by acid titration. Both D,L-lactamide and L-lactamide showed inhibition effects at high concentration although the pH of the reaction mixtures remained constant between pH 7.0 – 8.0 [section 5.3.3.1 – 5.3.3.2]. From these results, it can be concluded that inhibition of the amidase by the substrate (L-lactamide) was likely to be competitive. Inhibition of the amidase by D,L-lactic acid was studied for biocatalyst deactivation by acid titration and potential competitive inhibition. From the results obtained and discussed in sections 5.3.4.1 to 5.3.4.2, it can be concluded that D,L-lactic acid is a potential inhibitor of the amidase through acid titration (i.e. lowering of the reaction medium pH). Substrate inhibition could be avoided by choosing an optimum substrate concentration, while product inhibition could be avoided by controlling the reaction pH around 7.0.

The study of reaction rates for the production of D-lactic acid [section 5.3.1] showed that the highest reaction rates were achieved at a final substrate (D-lactamide) concentration of 40mM. Figure 5.1 also showed that the linear phase of the reactions occurred during the reaction time of 0 to 30 minutes, for concentrations between 10 and 60mM. It was concluded that the optimum substrate concentration for D-lactic acid production was 40mM and that the Michaelis-Menten kinetic constants should be measured over 30 minutes reaction time, under the experimental conditions. Studies of the effect of enzyme loading on the production of D-lactic acid showed that conversion of D-lactamide to D-lactic acid increased linearly with increasing enzyme concentration. Therefore it can be concluded that the amidase-catalyzed reactions do follow the Michaelis-Menten type kinetics. The effect of biocatalyst loading on conversion [section 5.3.5.2] also showed that, although the rate of conversion increased with increasing enzyme load, even high proportions (500mg biocatalyst/3.0ml reaction volume) of biocatalyst was not able to achieve 100% conversion. A number of factors could contribute to the inability of the biocatalyst to achieve higher conversions (section 5.3.5.1), including possible mass transfer limitations, biocatalyst inhibition, insufficient reaction time and biocatalyst deactivation. The reactions were carried out in batch reaction tubes, in which poor mixing could have resulted in mass transfer limitations. Also, it has been reported that most immobilized enzyme reactions are mass transfer limited, and that a high value of K_m indicates the presence of diffusion limitations [Bozhinova *et al.*, 2004]. Bozhinova *et al.* (2004) have studied the kinetic parameters for benzylpenicillin hydrolysis using free and immobilized penicillin amidase. The enzyme was immobilized by covalent attachment onto M-PVAE02, Sepharose and Eupergit C beads. Their results have showed that, compared to free enzyme,

the K_m values were 5.5, 200 and 300 times larger for enzyme immobilized on M-PVAE02, Sepharose, and Eupergit C respectively. In this study, although kinetic parameters for free amidase were measured with acrylamide as a substrate ($K_m = 13.9\mu\text{mol/ml}$, $V_{\text{max}} = 1.26\mu\text{mol/ml.min}$), while the kinetic parameters for immobilized amidase were measured with D-lactamide as a substrate, the K_m value for immobilized amidase was found to be about 40 times higher than that of the free amidase.

Immobilization of the amidase involved the use of glutaraldehyde as a cross-linking agent, and accumulation of the product inside the cross-linked glutaraldehyde-enzyme-carrier matrix could have resulted in biocatalyst inhibition. In Chapter 4, it was reported that the immobilized biocatalyst loses activity over time even at the optimum reaction temperature (50°C). Thus, thermal deactivation of the biocatalyst over time could have resulted in insufficient availability of biocatalyst to convert all or most of the substrate. The experimental time frame was 150 minutes, and it was suggested that over longer periods of time, a higher conversion could be achieved. Ultimately, one or all or a combination of some of the factors discussed above could be the cause of the observed conversion-time profiles under these experimental conditions.

In summary, a number of negative factors that could affect the productivity of the amidase in producing D-lactic acid have been discussed. However, factors such as biocatalyst thermal deactivation, diffusion limitations and residence time for maximum conversion, require in depth investigation in order to understand how they affect the amidase reaction. In the work reported in the next chapter, some of these factors were investigated by mathematical models, and thereafter the models were compared with experimental data.

Chapter 6 Development of a continuous reactor process for the production of D-lactic acid

6.1. INTRODUCTION

The previous chapters reported the production of the amidase, the studies of its biochemical properties and the development of an amidase biocatalyst through immobilization onto a suitable carrier. The primary aim of the work reported in this chapter was to demonstrate the potential of the amidase biocatalyst for an industrial application in the production of carboxylic acids. The production of D-lactic acid, which has a number of industrial applications [Chapter 5], was used as a demonstration process. Mathematical modelling of the reactor process gave a model that could be used in scale-up procedure and further application of this process, and for the production of other carboxylic acid products.

Based on the kinetic properties of the amidase and the immobilized enzyme properties studied in chapter 4, a small-scale packed-bed reactor was designed and developed. This process was used for the continuous production of D-lactic acid using the immobilized amidase biocatalyst, and the productivity of the process was measured based on product (D-lactic acid) formation. The mathematical fixed-bed reactor model was used to describe (under different operating conditions to the experimental conditions) and determine theoretical conditions (e.g. amount of biocatalyst and flowrate) capable of achieving the highest possible conversion of D-lactamide to D-lactic acid.

6.2. Description of the packed-bed reactor process

The reactor was a glass cylinder with flow inlet and outlet points at the bottom and top of the cylinder respectively. For temperature control, the cylinder was fitted with a glass heating/cooling jacket with flow inlet and outlet points at the sides of the reactor. For fixing the immobilized biocatalyst inside the reactor, the cylinder was fitted with a glass sieve near the bottom/inlet point. The top part of the cylinder was constructed to allow it to be removable for addition and removal of the biocatalyst, and for cleaning purposes. Figure 6.1 shows the drawing of the reactor, including a detailed drawing of the top part/outlet point of the reactor. The reactor design was based on the highest amount of biocatalyst that could be produced on laboratory scale, and not on the desired productivity, at this stage.

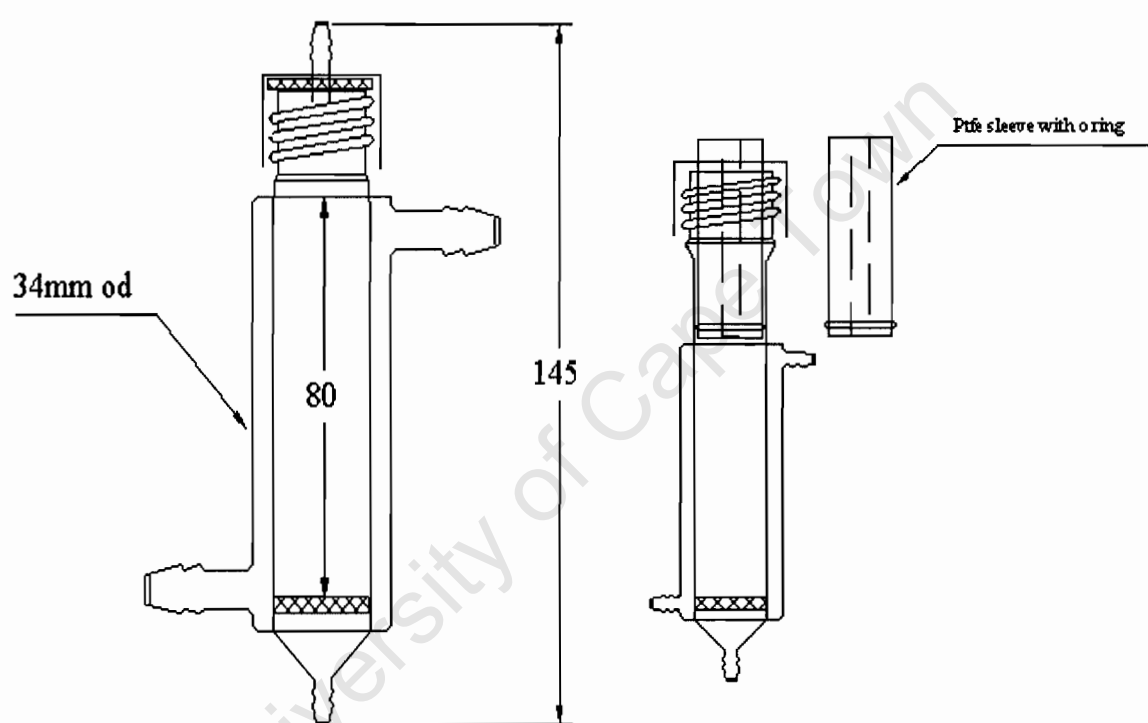


Figure 6.1: The packed-bed reactor used for the experimental production of D-lactic acid, using the amidase biocatalyst.

6.3. MATERIALS AND METHODS

6.3.1. Materials

The reactor, constructed from a borosilicate 3.3-type glass, was purchased from Glasschem, Stellenbosch (South Africa). All chemicals were purchased from Sigma-Aldrich (SA), Aldrich-SA and Fluka except for D-lactamide, which was purchased from Sigma-Aldrich (International). All solvents were purchased from Merck-SA. Eupergit CM was received as a gift from Röhm Pharma Polymers (Darmstadt, Germany) and the amidase biocatalyst was prepared as described in Chapter 4, section 4.2.4.

6.3.1. The experimental setup

The packed-bed reactor used was 145mm long, with a 100ml working volume, a 20mm internal diameter, and a 34mm external diameter. The bottom part/inlet point of the reactor was fitted with a 200 microns sinter disk for supporting the biocatalyst. The outlet point/top part was fitted with a Teflon (FTPE) plunger, which could be inserted inside the reactor to the required length, thereby adjusting the working volume based on requirement. The Teflon plunger was tightened using an attached O-ring screw cap. The reactor was constructed using borosilicate 3.3-type glass, while the plunger and O-ring were constructed using Teflon plastic.

Two Masterflex console drive pumps were used for the pumping of substrate and heating water. A water bath was used for temperature control of the heating water, and the reactor temperature was controlled by circulating the water (50°C) through the jacket. A 1L Schott bottle was used as a substrate vessel, placed in the water bath in order to maintain the substrate stream at 50°C. Another 1L Schott bottle was used as a product collection vessel, and this was fitted with a sampling valve. Figure 6.2 shows a detailed drawing of the experimental setup.

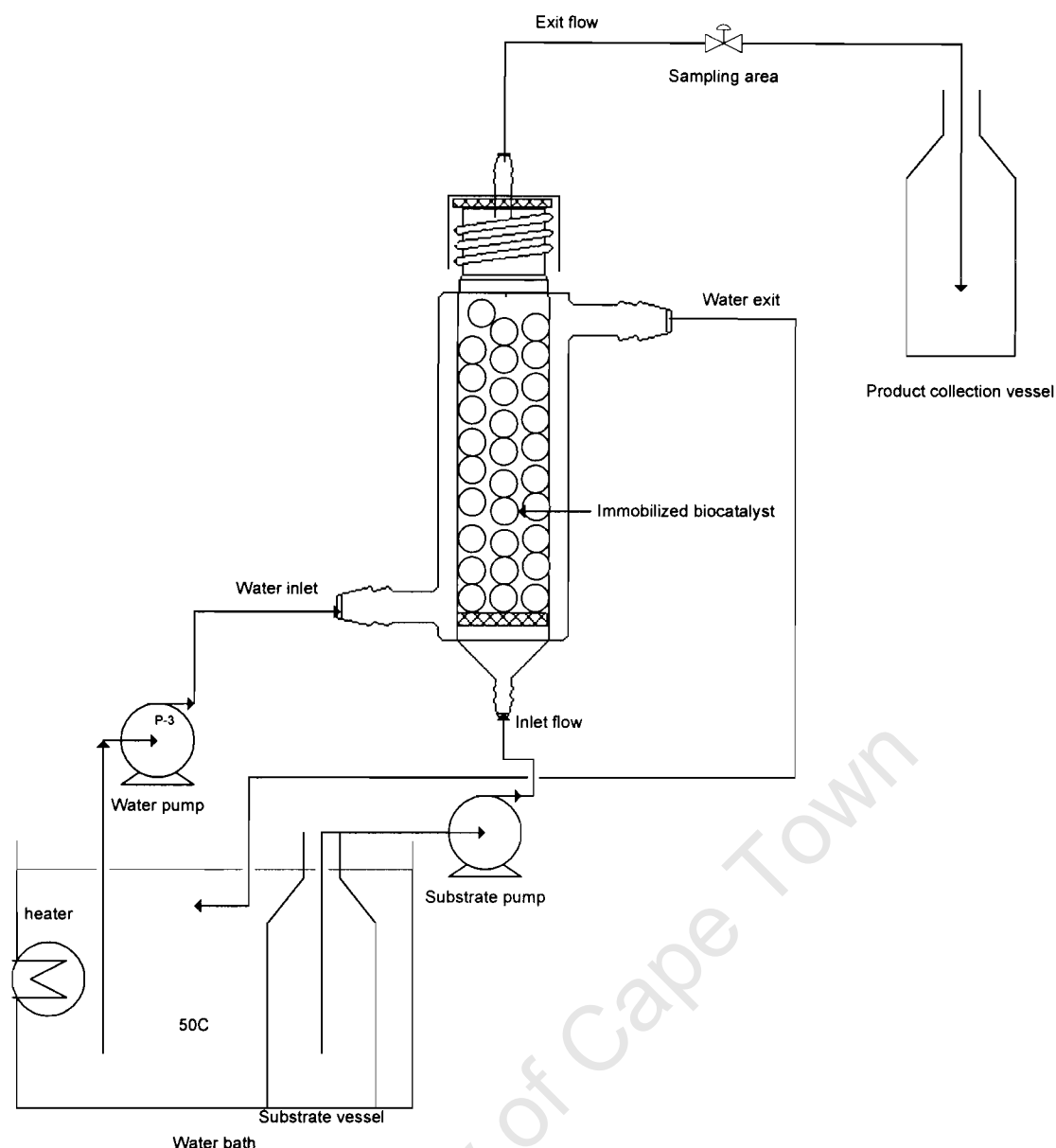


Figure 6.2: The experimental reactor setup used for the production of D-lactic acid, using the *Bacillus* sp. RAPc8 amidase biocatalyst.

6.3.3. Pump Calibration

The Masterflex console drive pumps were calibrated by pumping water (25°C), while measuring the time taken to fill a known volume container with the water. Pump settings were varied between 2.0 and 10.0, and a fixed volume of water (100ml) was pumped and then timed. The time measurements and the pump settings were used to plot a pump calibration curve.

6.3.4. Investigation of reactor flow properties

Flow properties of the reactor were investigated using 5.0g of wet non-immobilized Eupergit CM beads. The beads were packed inside the column (reactor), while distilled water (50°C) was pumped through the packing at different flowrates. The expansion of the bed was measured as a function of pump settings (i.e. flowrates). The bed expansion measurements were used to estimate the required flowrate (pump setting), to operate under packed-bed conditions, for the biotransformation experiment.

6.3.5. Continuous production of D-lactic acid in the packed reactor using the *Bacillus* sp. RAPc8 amidase biocatalyst

The experimental setup described in section 6.3.1 was used for the continuous production of D-lactic acid. 10mM of D-lactamide solution in 50mM phosphate buffer (pH 7.0), stored in the substrate vessel, was pumped from the bottom of the reactor through the biocatalyst packing, at selected flowrates. The exit stream, containing the product (D-lactic acid), was collected from the top of the reactor, into the product collection vessel. 1.0ml samples were withdrawn periodically, using the sampling valve, and these were used to determine the productivity of the process in terms of conversion of the substrate.

6.4. RESULTS

6.4.1. Pump Calibration

Pump calibration, performed as described in section 6.3.3, was done in order to determine the pumping rates of the pumps, at various settings. The relationship between pump settings and flowrate was found to be linear [Figure 6.3]. Therefore, using this relationship, it was possible to maintain a desired flowrate through the reactor, by choosing the appropriate pumping setting.

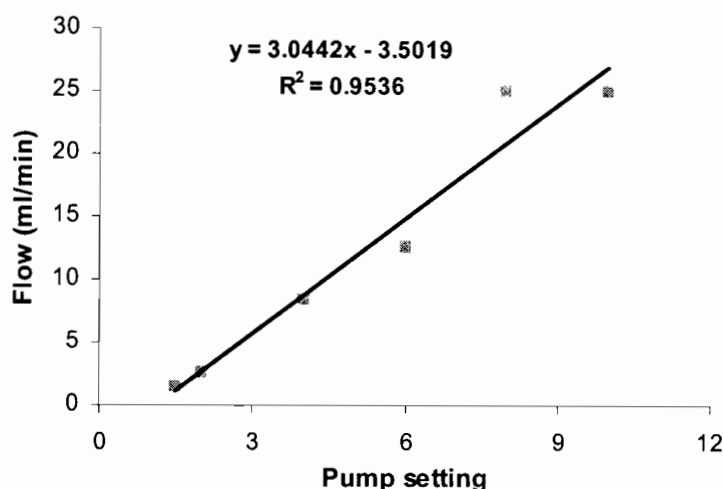


Figure 6.3: The pump calibration curve for the Masterflex console drive pumps.

6.4.2. Investigation of reactor flow properties

Expansion of a biocatalyst bed is an important property for determining the allowable range of flowrates for the desired reactor configuration (i.e. fixed-bed or fluidised bed). High flowrates often result in fluidisation of the biocatalyst bed, thereby changing the properties of the reactor to a fluidised bed [Coulson and Richardson, 1991]. The allowable range of flowrates was determined by measuring the height of the biocatalyst bed at different flowrates (i.e. pump settings). The relationship between pump setting and bed expansion was estimated by the curve shown in Figure 6.4. In this experiment, linear bed expansion was observed to occur at all flowrates above 3.0ml/min. However, it would be possible to operate at higher flowrates, if the top of the biocatalyst bed could be held fixed, by means of a rigid stopper or mash. The observed linear bed expansion will change the intended packed-bed reactor mode into a uniform expanded-bed mode. This will change reaction properties (e.g. mass transfer) inside the reactor, but will not affect reactor modelling, which will be based on overall mass balance of a packed column.

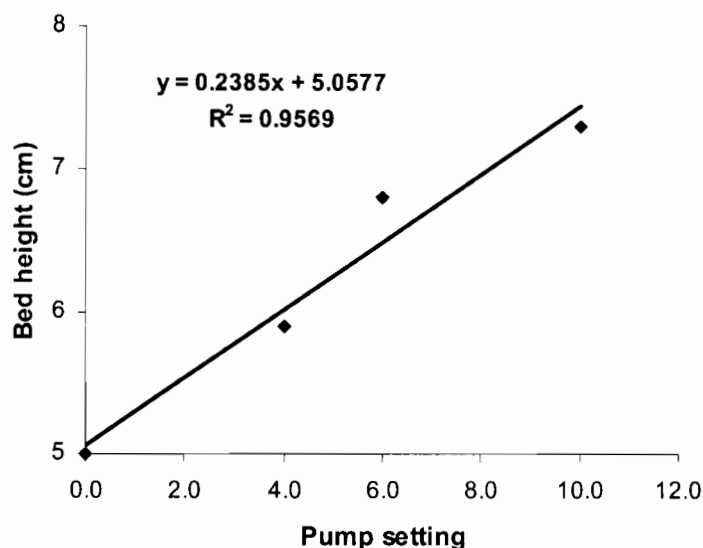


Figure 6.4: The relationship between pump setting and biocatalyst bed expansion for the amidase biocatalyst reactor process.

6.4.3. Continuous production of D-lactic acid in the packed reactor using the *Bacillus* sp. RAPc8 amidase biocatalyst

The intended packed-bed reactor system (described in section 6.3.5), which operated as a uniform expanded-bed reactor, was used for the continuous production of D-lactic acid, using the amidase biocatalyst. The amidase biocatalyst was prepared according the method described in section 4.2.4, and the kinetic properties of the biocatalyst (measured as described in section 5.7.3) are summarised in Table 6.1. The formation of D-lactic acid, described in terms of conversion by the

equation $\frac{[S_0] - [S]}{[S_0]}$, was measured over a fixed period of time. The inlet D-lactamide concentration into the reactor was fixed at 10mM, while the concentration of the D-lactamide at each measurement time period was determined using the ammonia detection assay [section 2.2.7]. The ammonia detection assay measures the concentration of the by-product (ammonia), which is stoichiometrically equivalent to the amount (moles) of the D-lactamide consumed. Therefore, the difference between the amount of D-lactamide consumed and the initial amount, gave the amount/concentration of the D-lactamide at each measurement time. Thus, the time-conversion profile for D-lactic acid formation was plotted and the conversion was found to decrease with increasing time, at both the flowrates investigated (i.e.

8.0ml/min and 2.0ml/min). The rate of decrease in conversion was found to be higher at the faster flowrate, and lower at the slower flowrate [Figure 6.5]. A number of factors may be accountable for this behaviour, including possible unsteady-state conditions, enzyme loss, biocatalyst deactivation, production inhibition and undesirable (high) flowrates due to reduced residence time. The highest conversion attained was estimated to be less than 30%. Mathematical modelling of the system would allow determination of the factors affecting the conversion, and thus the process could be optimised based on the results of the mathematical model. This is described in the section 6.4.5.

Table 6.1: Physical and kinetic properties of the amidase, used in the reactor.

Parameter	Value, units
Biocatalyst total weight	15.0 grams
Carrier particle size ^a	~200 µm
Carrier water content ^b	3.2 g water/g beads
Immobilized protein concentration ^b	0.0093 mg protein/mg beads
Biocatalyst specific activity ^b	103.5 U/mg
K _m	556.7 µmol/ml
V _{max}	3.97 µmol/ml.min

^a specified by Röhm Pharma Polymers (Darmstadt, Germany), Eupergit beads supplier.

^b estimated after amidase immobilization, using methods described in Chapter 4

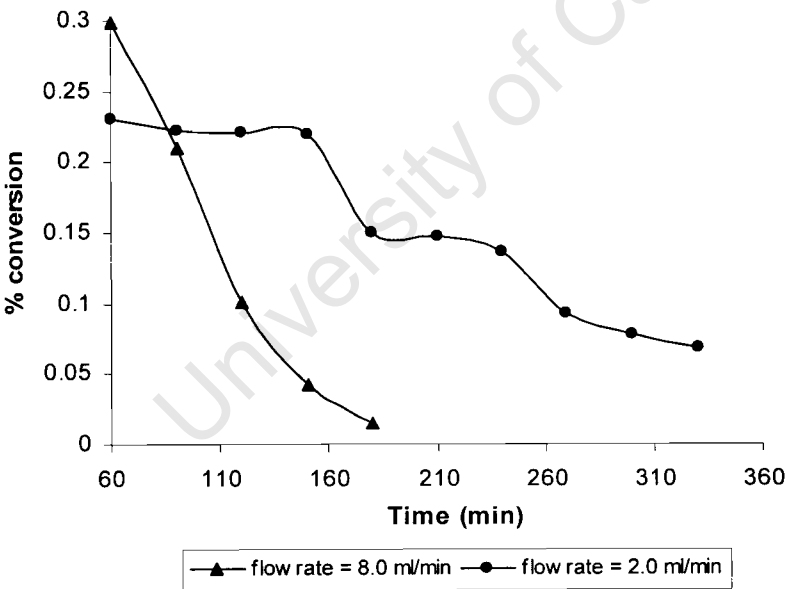


Figure 6.5: The time-conversion profile of the packed-bed reactor process at flowrates of 2.0 and 8.0ml/min.

6.4.4. Investigation of biocatalyst thermal deactivation

Enzymes can undergo an irreversible denaturation due to exposure to high temperatures [<http://www.Isbu.ac.uk/biology/enztech/temperature.html>]. The thermal stability of the immobilized amidase was studied and discussed in Chapter 4. Although the amidase was found to be thermally stable at 50°C, its specific activity was also found to decrease over time [section 4.3.2.3]. Biocatalyst thermal deactivation can take various forms (e.g. linear, exponential and etc.) [Fogler, 1999], and therefore, in order to include thermal deactivation into a mathematical model, the form of deactivation must be determined. Using the thermal stability data described in section 4.3.2.3, various forms of deactivation models were fitted to a curve. The curve was a plot of activity fraction, defined as the ratio of specific activity at time t [$a(t)$] to the initial specific activity [$a_0(t)$], versus incubation time. Two forms of deactivation, namely linear and exponential deactivation, were found to fit the data reasonably accurately. The mathematical representations for these forms of deactivation are shown by the following equations [Fogler, 1999]:

Linear deactivation: $a(t) = 1 - k_d * t$ equation 6-1

Exponential deactivation: $a(t) = \exp(-k_d * t)$ equation 6-2

where $a(t)$ = activity fraction at time, t

k_d = thermal deactivation constant

t = time

Therefore, based on Figure 6.6, the linear and exponential deactivation constants were found to be 0.002 and 0.0025 min⁻¹ respectively. In general, thermal deactivation of enzymes has been reported to follow exponential thermal decay [<http://www.Isbu.ac.uk/biology/enztech/temperature.html>], although the linear decay function fitted the data more accurately (i.e. R^2 value closer to 1.0) as compared to the exponential decay function. In the following sections where thermal deactivation was considered, an exponential thermal decay model was applied.

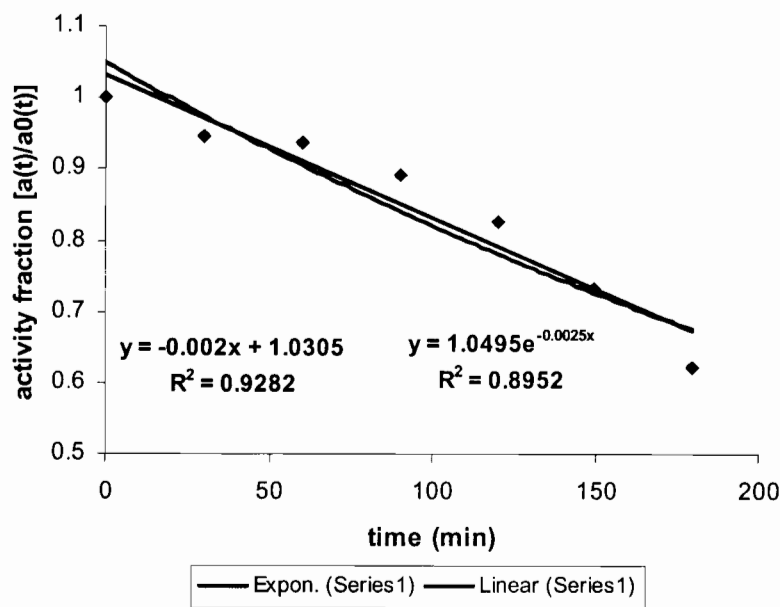


Figure 6.6: The thermal deactivation profile of the amidase biocatalyst.

6.4.5. Kinetic modelling of the packed-bed reactor

Previously, the yield in terms of conversion of the packed-bed reactor was found to be negatively affected by suspected biocatalyst deactivation, and possible unsteady state operation. Therefore, in the work reported in this section, a preliminary kinetic mathematical model was developed for the prediction and optimisation of the process yield, taking into account biocatalyst thermal deactivation, and possible unsteady-state behaviour. The kinetic model was based on the overall general mass balance equation for a packed column and Michaelis-Menten rate equation for a deactivating enzyme. The Michaelis-Menten equation for the rate of substrate utilization with biocatalyst deactivation is given by the following equation [Fogler, 1999];

$$-r_a = \frac{a(t) \cdot V_{max} \cdot C_a}{(K_m + C_a)} \dots\dots\dots\text{equation 6-3}$$

- where $-r_a$ = rate of substrate utilization
- $a(t)$ = rate of biocatalyst deactivation
- C_a = substrate concentration at time, t

The general mass balance equation for a packed column [Fogler, 1999] is given by the following equation;

$$F_{ao} - F_a + r_a \cdot V = \frac{dN_a}{dt} \dots\dots\dots\text{equation 6-4}$$

where F_{ao} = inlet molar flowrate of component a

F_a = exit molar flowrate of component a

V = volume of packing

N_a = moles of component a

By assuming a constant volume reactor, equation 6-4 can be modified as follows;

$$v_o \cdot C_{ao} - v_o \cdot C_a + r_a \cdot V = \frac{V \cdot dC_a}{dt} \dots\dots\dots\text{equation 6-5}$$

where v_o = volumetric flowrate

C_{ao} = initial molar concentration of component a

C_a = molar concentration of component a, at time t

Therefore, combining equations 6-3 and 6-5 and rearranging, the unsteady state fixed-bed reactor model is described as follows;

$$\frac{dC_a}{dt} = \frac{v_o}{V[C_{ao} - C_a]} + 1.049 \exp(-k_d \cdot t) \cdot \frac{V_{max} \cdot C_a}{K_m + C_a} \dots\dots\dots\text{equation 6-6}$$

Polymath 5.1 was used to solve the ordinary differential equation [6-6]. The conversion, defined as $\frac{C_{ao} - C_a}{C_{ao}}$, at the experimental flowrates of 2.0 and 8.0ml/min, was found to first increase with time, until maxima at around 20 and 7% respectively. Above the maximum, the conversion decreased with increasing time [Figure 6.7]. In order to determine the extent to which biocatalyst thermal deactivation affects the conversion in the reactor, the model, equation 6-6, was solved with the absence of thermal deactivation. In this case, the conversion was found to increase to a maximum (~23%), and thereafter maintained the same maximum conversion over time.

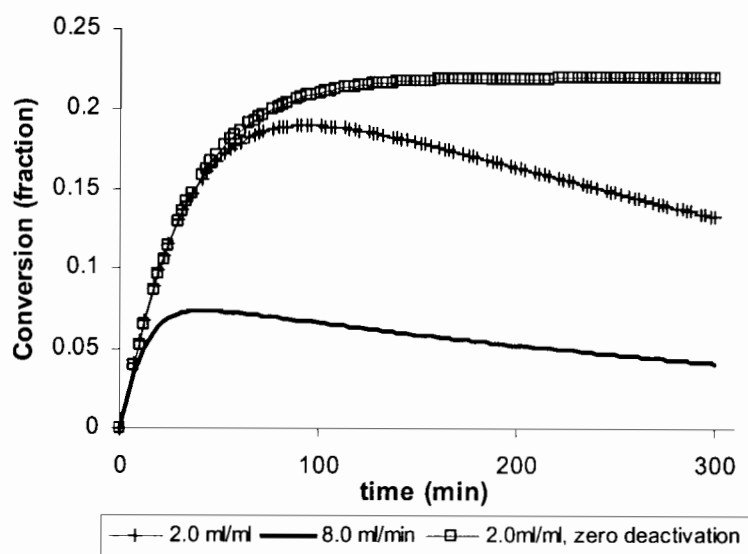


Figure 6.7: The conversion profiles of D-lactic acid production based on the unsteady state packed-bed reactor model, using the *Bacillus* sp. RAPc8 amidase biocatalyst.

If the inlet and outlet flowrates through the fixed-bed were controlled, the process would operate under steady-state. Therefore, the term on the left of equation 6-6 would disappear and the new equation would describe the packed-bed reactor under steady state conditions. A steady-state model was derived [Table 6.2] and used to determine the relationship between steady and unsteady state behaviour based on conversion in the reactor process. Figure 6.8 shows the plots of the conversion profiles obtained under both ideal (i.e. with no biocatalyst deactivation) and biocatalyst thermal deactivation conditions. Under conditions with no biocatalyst deactivation, the conversion was maintained at a constant value of about 22% throughout, whereas the conversion decreased with increasing time under biocatalyst deactivation conditions [Figure 6.8]. Thus, the models clearly show that biocatalyst deactivation affects the conversion in the reactor process in a negative manner, irrespective of steady or non-steady state in the reactor.

Although the biocatalyst deactivation model fits the experimental results discussed in section 6.4.3 closely, the question should be asked as to whether biocatalyst deactivation is, in fact, the main contributor to the time-conversion profile shown by figure 6.5, rather than factors such as diffusion limitation, enzyme loss and possible biocatalyst inhibition. In order to answer this question, the following was considered:

It is known that biocatalyst deactivation is generally an irreversible process [http://www.Isbu.ac.uk/biology/enztech/temperature.html]. Thus, the biocatalyst used in the packed-bed process should exhibit little or no further activity if was to be re-used in another biotransformation. A batch reaction was conducted using biocatalyst withdrawn from the packed-bed reactor, at the end of the experiment discussed in section 6.4.3, and it was found that the amidase biocatalyst indeed exhibited no activity towards D-lactamide.

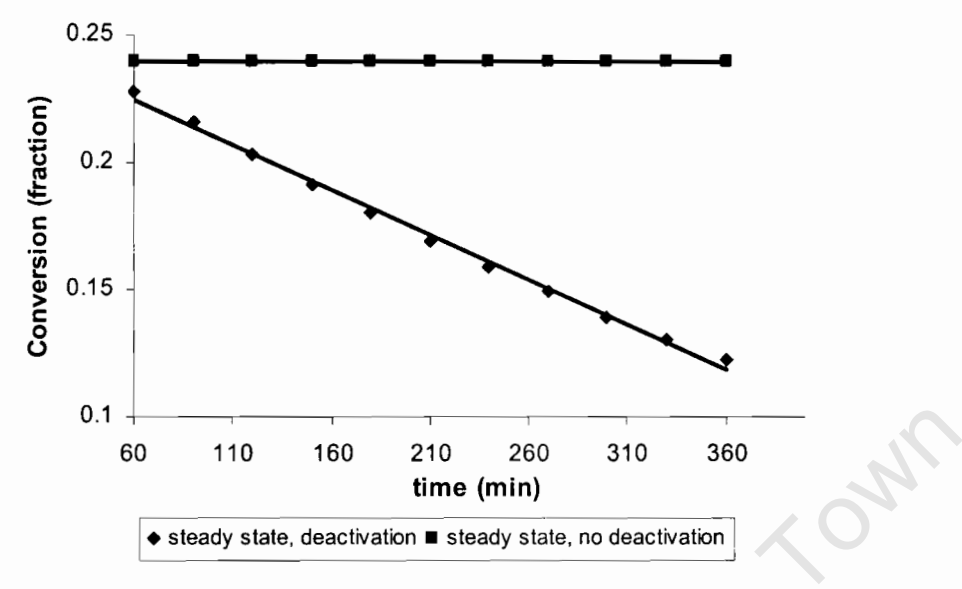


Figure 6.8: The conversion profiles of D-lactic acid production based on the steady state packed-bed reactor model, using the amidase biocatalyst.

Table 6.2: Snapshot of the excel spreadsheet used for solving the steady state packed-bed model at different time periods.

Parameter	No deactivation	Thermal deactivation	Units
K_m	556.70	556.70	$\mu\text{mol/ml}$
V_{\max}	3.97	3.97	$\mu\text{mol/ml.min}$
v_o	2.50	2.50	ml/min
V	100.50	100.50	cm^3
C_a (guess)	7.84	7.79	$\mu\text{mol/ml}$
a (t)	0.97	-	dimensionless
r_a	0.06	0.05	$\mu\text{mol/ml.min}$
C_a (calculated)	7.84	7.80	$\mu\text{mol/ml}$
conversion	0.22	0.22	dimensionless

6.4.6. Comparison between experimental data and steady-state model

Comparison between experimental data and the steady state models was done by plotting the experimental data and mass balance model on the same set of axes [Figure 6.9]. The experimental data, at 2.0ml/min flowrate, were found to reasonably follow the steady-state biocatalyst deactivation model. At 8.0ml/min, the model under-predicted conversion initially and further under-predicted the rate of enzyme deactivation. In section 6.4.2, it was shown that linear bed expansion occurred at all flowrates above 3.0ml/min, and hence the reactor operated as a uniform expanded-bed reactor rather than a fixed-bed reactor. The kinetic model developed was not based on the behaviour of the biocatalyst bed (i.e. packed-, expanded-, or fluidised-bed). It should be noted that, amongst other things, the behaviour of the biocatalyst bed affects the productivity and operation of the reactor process.

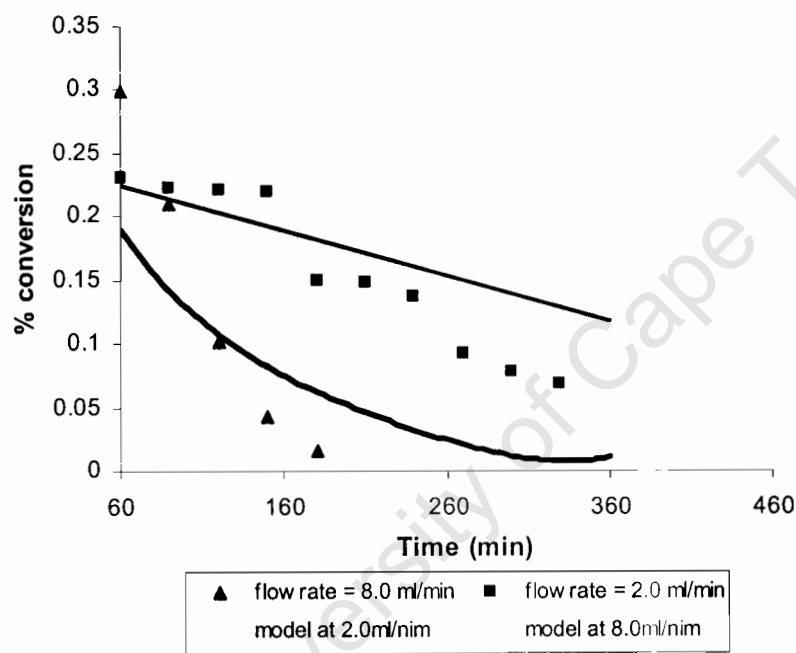


Figure 6.9: Comparison between experimental conversion profile and model-predicted conversion profile for D-lactic acid production using the amidase biocatalyst.

6.4.7. Theoretical optimisation of the packed-bed reactor productivity

In the amidase packed-bed reactor model described in section 6.4.5, flowrate and the amount of biocatalyst are the operating parameters that can be varied in order to improve the productivity of the process. The other parameters, including Michaelis-Menten constants and the thermal deactivation constant, are all constant parameters defined by the properties of the biocatalyst. In the previous investigation (section 6.4.5), the model showed that the slower the flowrate, the better the substrate conversion in the process. Also, increasing the amount of biocatalyst present would improve the conversion in the reactor, due to the increased probability of the substrate encountering a biocatalyst active site. Therefore, these two parameters were varied in order to achieve the highest possible conversion, using the model described by equation 6-6. The pump was set to the lowest rate possible (i.e. 0.5ml/min), while the entire working volume of the reactor [section 6.2] was assumed to be packed with the biocatalyst. Figure 6.10 shows the modelled time-conversion profile under these conditions. The conversion increased with time, until a maximum around 50%, after which the conversion decreased with increasing time [Figure 6.2]. The decrease in conversion can be attributed to biocatalyst deactivation, as discussed in previous sections. Therefore, the experimental setup (i.e. reactor size and pumps) is capable of converting a maximum of approximately 50% of the substrate (D-lactamide), under the substrate concentration used. Thus, a larger reactor volume, a more stable biocatalyst and smaller pumps able to pump at flowrates below 0.5ml/min, could improve the conversion. Changing the inlet concentration of the substrate would not change the conversion per pass, as the ratio of $\frac{C_{ao} - C_a}{C_{ao}}$ would remain constant; but it would increase the overall conversion.

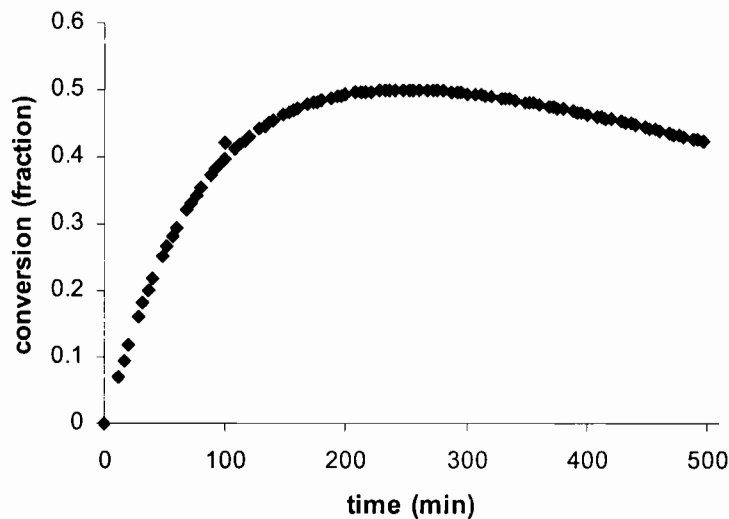


Figure 6.10: The theoretical time-conversion profile for D-lactic acid production, under optimised reactor conditions, based on the unsteady state reactor model, using the amidase biocatalyst.

6.5. GENERAL DISCUSSION AND CONCLUSIONS

Pump calibration was necessary to determine the relationship between pump settings and flowrate. From the results discussed in section 6.4.1, it can be concluded that there was a strong linear relationship between the Masterflex pumps settings and the flowrate. The packed-bed experimental setup was bound by flowrate restrictions due to the free-packing of the biocatalyst (i.e. unfixed top of the biocatalyst bed). Based on the fact that the biocatalyst beads undergo fluidisation at flowrates above 10ml/min, it was concluded that the 10ml/min was the maximum allowable flowrate for operation as either a packed-bed or a uniform expanded reactor, under the experimental setup.

The yield of the packed-bed process, defined in terms of conversion, was influenced by certain operating parameters and properties of the amidase biocatalyst, including flowrate, amount of biocatalyst, and biocatalyst deactivation. In order to achieve a desired conversion, a combination of these operating parameters should be optimised.

The ratio of biocatalyst volume to substrate volumetric flowrate (i.e. $\frac{V}{v_o}$)

[equation 6-6] is known as the residence time (i.e. the time spent by the substrate inside the reactor) [Fogler, 1999]. In general, the higher the residence time, the higher the productivity and operating costs of the reactor, due to increased reaction period and operating time. By choosing a cost effective amount of biocatalyst and flowrate, which could produce a desirable conversion of the substrate, the residence time could be optimised. The production of D-lactic acid, using 5.0g of amidase biocatalyst and the flowrates 2.0 and 8.0ml/min, showed that approximately 30% was the highest conversion achievable in the experimental packed-bed reactor, in which the residence time was approximately 20 minutes. The decrease in conversion with time was higher at faster flowrates and lower at slower flowrates. This result clearly shows that at slower flowrates, where the substrate spends more time inside the reactor, each substrate molecule has an increased probability of binding into an active site of the biocatalyst, and thus gets transformed into product. Based on these results, it can be concluded that slower flowrates would be preferred in order to improve conversion in the process. The model described in section 6.4.7 predicted that the highest possible conversion (~50%) could be achieved with 10.0g of biocatalyst and 0.5ml/min flowrate, in which the residence time would approximately be 125 minutes, under the experimental setup used.

In a biocatalytic process, the biocatalyst plays a central role, and it must be protected against possible deactivation. The results shown in section 6.4.4 indicated that thermal deactivation of the amidase biocatalyst fitted both linear and exponential decay models reasonably closely. Based on the fact that enzymes generally follow an exponential thermal decay, it was decided that the kinetics of the biocatalyst would be modelled based on exponential thermal deactivation model. The packed-bed kinetic models (both steady- and unsteady-state), have showed that the thermal deactivation of the amidase resulted in a decrease in conversion with increasing time, whereas a constant conversion was maintained throughout, under ideal conditions (i.e. with no biocatalyst deactivation) [section 6.4.5]. It can be concluded that biocatalyst deactivation severely affects the yield of the process. Also, the two models gave similar results, even though they were different mathematically, and it can also be concluded that the unsteady state assumption, which lead to the development of an unsteady reactor model, can be neglected, and also since the experimental data in section 6.4.3 showed that the measurements taken were under steady-state conditions.

It has to be noted that the first measurement (sample) was taken approximately 1 hour after the start of the experiment, which could have given the process sufficient time to reach steady state, as shown in figure 6.9. In summary, biocatalyst deactivation was reported to be one of the negative factors influencing conversion in the packed-bed reactor process [section 6.4.4]. It is known that as the biocatalyst deactivates, the number of active enzyme molecules available for substrate binding decreases. The rate of substrate conversion in the reactor process decreased with increasing time, due to this effect. This effect resulted in the behaviour of the time-conversion profiles observed from both the experimental and mathematical data [sections 6.4.3 & 6.4.5]. Although high temperatures generally result in higher reaction rates, factors such as biocatalyst deactivation mean that the conversion is compromised.

Reactor design mainly involves the development of a reactor capable of achieving the desired conversion. In section 6.2, it was shown that the packed-bed reactor was developed based on the highest amount of biocatalyst that could be produced on laboratory scale. The results discussed in section 6.4.7 showed that the experimental setup was capable of converting a maximum of 50% of the substrate. Therefore, the low yield of the packed-bed process could be attributed mainly to reactor design restrictions. Since the dynamics and properties of the reactor process are known, an appropriate mathematical model should be used to design another packed-bed reactor and also to investigate conditions which could result in the conversion of 100%.

As discussed earlier, the kinetic models of the reactor process have predicted that conversion would increase to a maximum, and thereafter decrease with increasing time [section 6.4.5]. Experimental data has also showed that, at steady state, the conversion decreased with increasing time [section 6.4.3]. The validity of the models was determined as discussed in section 6.4.7. The close relationship between the experimental data at the flowrate 2.0ml/min and the kinetic model [Figure 6.9] was due to the fact that the model itself was developed using the 2.0ml/min flowrate, and therefore it was not surprising that experimental data at 8.0ml/min flowrate was not as closely related to the model. Also, according to Figure 6.9, the experimental rate of decrease in conversion with time was faster than that predicted by the model. This result could be attributed to either model or experimental error, or to the influence of other factors, including diffusion limitations and biocatalyst inhibition.

This study was limited to demonstrating the potential ability of the amidase biocatalyst to produce D-lactic acid continuously, and modelling the process using a simple mathematical model. However, diffusion limitations, which are often complex, should be investigated by measuring the mass transfer coefficient of D-lactamide across the “enzyme-carrier matrix”, formed by the cross-linking agent (glutaraldehyde). The rate of substrate and/or product diffusion through the “stagnant layer” (enzyme-carrier matrix) could be fast or slow, and therefore the overall reaction would be diffusion limited, in the case of slow diffusion rate, or kinetically controlled, in the case of fast diffusion rate [<http://www.isbu.ac.uk/biology/enztech/diffusion.html>]. A simple mathematical equation for testing whether a reaction is kinetically controlled or diffusion limited is called the Damköhler number [<http://www.isbu.ac.uk/biology/enztech/diffusion.html>]. The Damköhler number, also known as the substrate modulus, is a dimensionless ratio of the reaction velocity to the transport velocity, and it is defined as follows;

$$\mu = \frac{V_{\max}}{k_L \cdot K_m} \dots\dots\dots \text{equation 6-7}$$

where μ = Damköhler number

V_{\max} = the maximum rate of reaction for a given enzyme concentration

k_L = mass transfer coefficient

K_m = Michaelis constant

Therefore at low substrate modulus (i.e. $\mu \leq 1.0$), the overall reaction is under the kinetic control of the enzyme, whereas at high substrate modulus (i.e. $\mu \gg 1.0$), the overall reaction is diffusion limited. Therefore for future work, diffusion limitations could be tested using this procedure, thereby allowing it to be either included or excluded in the mathematical model, depending on the results.

In summary, the yield of the packed-bed is affected by a number of operating parameters, some of which can be optimised. Other parameters including biocatalyst deactivation cannot be optimised but can be controlled by choosing favourable operating conditions for the biocatalyst.

Chapter 7

General Conclusions

7.1. INTRODUCTION

This study was focused on biochemical studies of a thermostable amidase and developing it into a potential industrial biocatalyst for the production of a target product. In the work reported in this chapter, the results obtained and discussed in the previous chapters have been considered and their implication discussed with regard to the aims and objectives set out in chapter 1. A number of recommendations have also been made based on these results and anticipated future work.

The investigations reported in this study have showed interesting results, some of which were expected, while others were not. Studies of the biochemical properties of the amidase, for example, showed that the enzyme has properties similar to the other enzymes belonging to its class, namely the wide spectrum amidases, with the exception that this amidase also exhibited enantioselectivity towards lactamide. Unexpectedly, the development of an amidase biocatalyst through immobilization resulted in a thermally more stable biocatalyst.

7.2. PRODUCTION AND BIOCHEMICAL CHARACTERIZATION OF THE FREE AMIDASE

The production of amidases by recombinant microorganisms involves steps including cloning into a host microorganism, and induction of the expressed amidase gene. Cameron (2001) cloned and over-expressed the amidase from *Bacillus* sp. RAPc8 into *E. coli* BL21 pNH223 pLySs, and also studied some biochemical properties of the enzyme. Here, the enzyme was produced in shake-flask culture, with high level expression using IPTG as an inducer. In the present study, the first objective was to produce the amidase in significantly larger amounts and to purify it for experimental purposes. The amidase was produced with high level expression in both shake flask and 7.5L commercial fermentor (BIOFLO 110 series) cultures. The large-volume production of the amidase in the BIOFLO 110 series fermentor resulted in significant

biomass yields (~0.5g/L of broth) and the extraction of a highly active enzyme, although optimisation of the fermentor operating parameters could result in a further improvement in the growth of the recombinant *E. coli* strain, and hence increased amounts of the enzyme. Furthermore, the novel heat-treatment purification of the amidase, developed by Cameron (2001), resulted in a sufficiently pure enzyme for experimental applications. The ability of the heat-treatment purification step to remove up to 70% of unwanted proteins showed that a combination of this method with other known purification methods (e.g. ammonium sulphate) could result in an enzyme sufficiently pure for commercial applications.

Due to the novelty of the amidase studied in this report, the second and third objectives were to study the biochemical properties of the amidase in terms of optimum operating conditions, and substrate- and chiral-selectivity. The studies of these properties revealed that, similar to the findings by Cameron (2001), the amidase operates optimally at a temperature of 50°C and pH 7.0 and that the substrate specificity of the amidase was limited to the hydrolysis of short- and mid-length aliphatic amides such as acrylamide, acetamide, lactamide and propionamide. The chiral selectivity of the amidase, studied using pure isomers of D- and L-lactamide, was D-specific in the case of lactamide. Literature survey revealed that amidases possessing such substrate specificity are well known and belong to the class of wide spectrum amidases. Until other substrates are used for further studies of the amidase chiral selectivity, it is not known whether the amidase is completely D-specific towards chiral substrates or whether the results were only applicable in the case of lactamide.

Based on the discussion of substrate specificity and chiral selectivity above, it can be concluded that the amidase can be utilized for the production of optically pure short- and mid-length aliphatic amides. With modern molecular techniques, it could be possible to alter the specificity of the amidase thereby producing an enzyme capable of catalyzing the hydrolysis of either aliphatic or aromatic amides, or both. Although, potential loss of amidase activity due to microbial contamination can be reduced by performing reactions at higher temperatures, enzyme activity can also be lost due to the presence of chemical inhibitors. Therefore, the fourth objective was to investigate the effects of various chemical compounds including sulfhydryl and chelating

reagents, heavy metal ions and water-miscible organic co-solvents, on the activity of the amidase. The amidase was found to be severely inhibited by the presence of heavy metals such as Hg^{2+} , Cu^{2+} and Co^{2+} , and organic co-solvents such as DMSO, DMS and mercapto-ethanol. Alcohols such as ethanol and n-butanol reduced the activity of the amidase by approximately 50%, while DTT enhanced the activity of the amidase by approximately 150%. Therefore, the results support the conclusion that sulfhydryl groups play a crucial role in the catalytic mechanism of the amidase. The presence of inorganic salts (e.g. NaCl), chelating reagents (e.g. EDTA) and protease inhibitors (e.g. PMSF) did not affect the activity of the amidase. Therefore, although the use of amidases in organic solvents has enormous potential and a number of advantages, a good polar-solvent, in which the amidase could retain stability and high activity, is yet to be found. It can also be concluded that in biotransformation reactions catalyzed by the amidase, heavy metals with sulfhydryl characteristics and high concentrations ($>5\text{v/v}\%$) of organic co-solvents should be avoided, while DTT could be used to enhance the catalytic activity of the amidase.

In summary, the results discussed above suggest that the thermostability of the amidase, which could potentially reduce purification costs, increase productivity due to higher reaction rates, and reduce chances of microbial contamination, together with its broad substrate specificity and its chiral selectivity, make it a potentially valuable industrial biocatalyst for the production of various optically-pure aliphatic carboxylic acids.

7.3. IMMOBILIZATION AND BIOCHEMICAL CHARACTERIZATION OF THE IMMOBILIZED BIOCATALYST

Stabilisation of biocatalysts by immobilization, both of whole cells and isolated enzymes, has been in existence for a number of years. A significant number of immobilization methods have been developed, and are being improved continuously. In general, immobilization methods are developed with the primary objective of creating an industrially viable biocatalyst.

Very few studies have reported the immobilization of wide spectrum amidases using different techniques and thus, the fifth objective of this study was to develop an

industrially viable biocatalyst through the immobilization of the amidase on a suitable carrier. Immobilization of the amidase by entrapment in a polyacrylamide gel, and covalent attachment onto Eupergit C beads at 4°C, resulted in low protein binding and low immobilized activity. The unfavourable immobilization temperature (4°C) and a potentially inhibitory chemical environment, including high concentrations of acrylamide and the presence of chemicals compounds such as SDS and ammonium persulphate, could be attributable for these results. High protein binding yield and high immobilized activity were observed when the amidase was immobilized on Eupergit C beads, with subsequent cross-linking using glutaraldehyde, at room temperature. Therefore, it can be concluded that by choosing a suitable carrier, avoiding the use of potentially inhibitory chemical compounds, enhancing protein binding through the use of a cross-linking agent and immobilizing under favourable conditions (i.e. temperature), an industrially-applicable aliphatic amidase biocatalyst was developed.

Generally, immobilized biocatalysts show modified biochemical properties. The sixth objective was to study the biochemical properties of the amidase immobilized on Eupergit C beads, in terms of optimal reaction temperature and pH, and thermal stability. The optimal operating conditions of the amidase (i.e. reaction temperature and pH) were found to be unchanged after immobilization, whereas its thermostability was improved through its increased stability at high temperatures, and high and/or low reaction pH. The implication of these results is that the amidase biocatalyst would be able to maintain higher activity under generally fluctuating industrial conditions.

7.4. DEVELOPMENT OF A CONTINUOUS D-LACTIC ACID PRODUCTION PROCESS

The seventh objective of this study was to choose a target product for potential industrial production using the amidase, based on the economic importance of the product. The market for lactic acid has been reported to be growing annually, due its recent application in the medical field, and furthermore, biologically produced lactic acid, as with many other products, is more preferred for food and pharmaceutical applications. Therefore, based on the market for lactic acid and the ability of the amidase to catalyze its production, lactic acid was chosen as a target product.

The eighth objective was to develop a preliminary continuous reactor process for the production of D-lactic acid, and although the novel research into the production of D-lactic acid using the proposed amide biotransformation method was preliminary, the amidase biocatalyst has shown great potential, based on several potential advantages. For example, the current commercial production of lactic acid is carried out in a typical batch reactor process, whereas the proposed amide biotransformation method could operate continuously. Furthermore, the proposed method could result in the use of an immobilized biocatalyst capable of being recycled and re-used several times.

The packed-bed reactor process that was developed resulted in a time-conversion profile that decreased with time, and in which 30% was the highest conversion achieved, under the experimental conditions used. The results showed that the low productivity and the decrease in conversion with time could be due to factors including mass transfer limitation, biocatalyst inhibition, and deactivation and inadequate reaction conditions (i.e. residence time, mixing).

Simple kinetic modelling of the reactor process (the final objective of this study) resulted in a model that indicated that thermal deactivation of the amidase biocatalyst was a contributor to the decreasing conversion-profile. Although only biocatalyst deactivation was taken into account in the development of the kinetic model, other factors could also have played a significant role in the behaviour of the system. It can be concluded that, in order to fully understand the factors affecting conversion in the packed-bed reactor, all possible factors must be included in the mathematical equation, which should then be solved and compared with experimental data. Although, the preliminary fixed-bed reactor process resulted in low yield of lactic acid, the kinetic model has also showed that the reactor size was, in fact, limited to a maximum of 50% conversion, based on reactor size and the experimental conditions. Taking this factor into account, the conversion of 30% was significant, since the reactor operating parameters (such as flowrate) were not optimised, and the biocatalyst underwent thermal deactivation over the time of operation.

The mathematical model, itself, was found to closely describe the experimental data at the flowrate of 2.0ml/min even though factors such as diffusion limitations and possible inhibition were not included. Also, the main assumptions of the model were

that the flow was laminar, the biocatalyst was uniformly distributed inside the reactor, and the substrate stream was uniformly dispersed across the biocatalyst bed (i.e. with no channelling of flow). These assumptions are often not true as flow properties do change, and it was also observed that in the actual experiments, some flow properties did change (i.e. flow changing between laminar and turbulent, and some degree of channelling occurring) during the reactor operation. Based on the fact that the simple model developed described the experimental data accurately, it can be concluded that the packed-bed reaction process could be modelled more accurately using a similar relatively simple model, which could then be used to further optimize the productivity of the experimental process.

In summary, the amidase biocatalyst, under optimised packed-bed conditions, could be used to produce D-lactic acid, and probably other carboxylic acids, potentially at industrial scale. If a scaled-up packed-bed process was to be developed for the amidase biocatalyst, the negative factors that affect biocatalyst activity (such as possible inhibition and deactivation) must be monitored and controlled. In a potential industrial application of this process, a disadvantage would be the envisaged cost of producing the amidase biocatalyst continuously. This would be necessary due to the irreversible thermal deactivation, instead of the biocatalyst being used several times to reduce costs, irreversible thermal deactivation would only allow the biocatalyst to be used once.

The current industrial processes producing lactic acid (i.e. chemical synthesis and carbohydrate fermentation) have more disadvantages than the proposed biotransformation method. These include batch processing, and recovery and separation costs that account for about 50% of the production costs [chapter 5]. Therefore, based on these observations, it can be postulated that the cost of biocatalyst production could probably be justified, based on the envisaged cheaper price of Eupergit C beads and fermentation broth chemicals as compared to the capital and operating costs of separation equipment (e.g. distillation columns, absorbers). Although work still needs to be done to improve the efficiency of the fixed-bed reactor process, the preliminary studies of the proposed method have shown encouraging results.

7.5. RECOMMENDATIONS

Based on all the results obtained from this research, the following recommendation can be made:

- Large volume fermentation of the recombinant *E. coli* strain encoding the amidase gene, using commercial fermentors should be conducted. Further studies of the factors affecting growth would facilitate optimisation of biomass yield and enzyme yield, and control of the fermentation.
- In a large-scale process, recovery of the intracellular amidase from the *E. coli* cells by cell disruption also requires further development, improvement and standardization to give a cost effective method. The cell disruption method used in this study (ultra-sound cell disruption), would be inapplicable at industrial scale, and therefore other industrially applicable methods should be developed.
- Maintaining the optimal biotransformation reaction conditions at industrial scale is critical, due to the high costs associated with large-scale operations (e.g. pumps, steam for temperature control). Therefore, further research should be done in determining fixed-bed operating conditions (e.g. temperature, flowrate) to achieve high productivity from the biocatalyst, thereby improving product formation and reducing biocatalyst waste, and hence production costs.
- Although further studies of the packed-bed reactor process could result in improved lactic acid productivity, other reactor configurations could perhaps achieve higher productivity. Therefore, it is recommended that other reactor-types (e.g. stirred-tank, fluidised-bed and membrane bioreactor) be investigated.

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APPENDICES

Appendix A: Culture medium and Reagents

A1. **Amidase liquid culture medium**

Add 16g of nutrient broth in 1000ml distilled deionised water. Autoclave
Add 100µg/ml of carbenicillin disodium salt solution.

A2. **Amidase plate culture medium**

Add 16.0 and 12.0g of nutrient broth and nutrient agar in 1000ml distilled deionised water respectively. Autoclave and cool down to room temperature. Add 100µg/ml of carbenicillin disodium salt solution. Pour into sterile plates.

A3. **Reagent A of the ammonia detection assay**

Add 5.55g (0.59M) phenol and 1mM sodium nitroprusside in 100ml distilled deionised water. Foil to protect from light and store at 4°C.

A4. **Reagent B of the ammonia detection assay**

Add 0.11M sodium hypochlorite and 8.0g (2.0M) sodium hydroxide in 100ml distilled deionised water. Store at 4°C.

A5. **FeCl₃ solution for acyl transfer reactions**

Dilute 32% HCl (~13M) to a final concentration of 0.65M using distilled deionised water. Add FeCl₃ granules to a final concentration of 356mM. Mix and store at room temperature.

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A6. Bradford’s reagent for protein determination

Use protein dye reagent from Sigma-Aldrich (Catalogue No. B6919) or prepare by dissolving 100mg of Coomassie blue G250 in 50 ml of 95% ethanol, then mix with 100ml of 85% phosphoric acid and make up to 1.0L with distilled water.

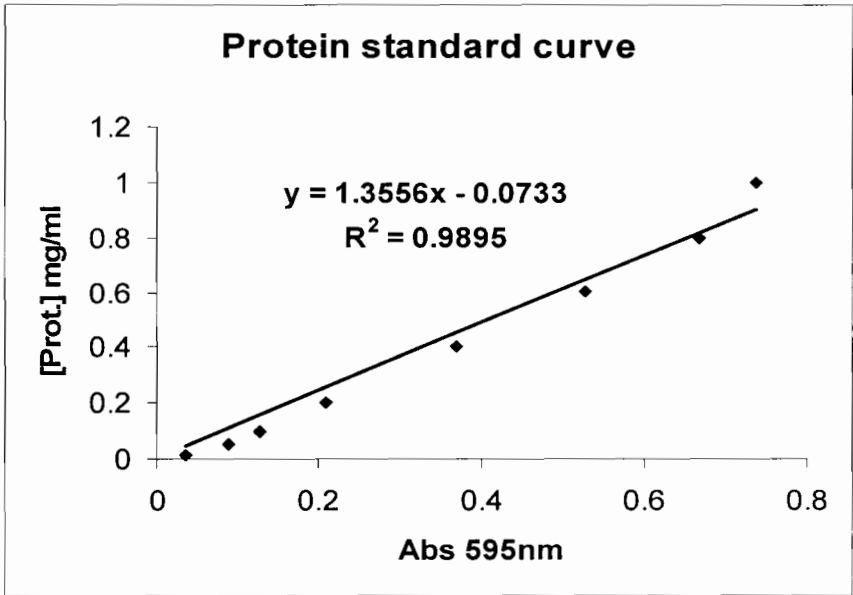
Appendix B: Standards and Buffers

B1. Bovine serum albumin (BSA) standard

Prepare a stock of 1mg/ml BSA solution by dissolving 10mg BSA in 10ml distilled deionised water. Using the stock solution, prepare various concentrations of BSA standards as follows:

BSA standard conc. (mM)	Volume of 1mg/ml stock added (ml)	Volume of distilled water added (ml)
0	-	10.0
0.2	2.0	8.0
0.4	4.0	6.0
0.6	6.0	4.0
0.8	8.0	2.0
1.0	10.0	-

In 1ml cuvettes, add 1ml of Bradford’s reagent (A6) and then add 20µl of the BSA standard in each cuvette. Leave for 5 minutes at room temperature and thereafter measure absorbance at 595nm, using a spectrophotometer.



B2. Ammonium chloride standard

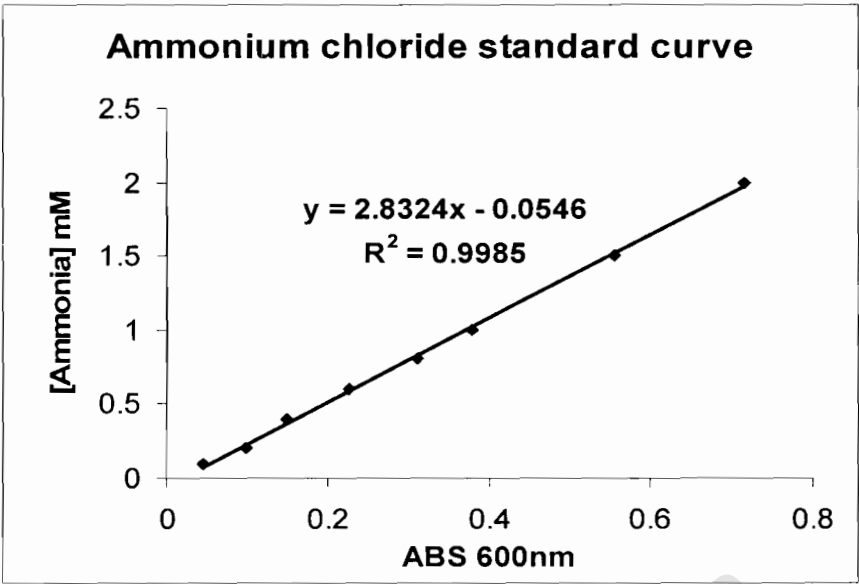
Prepare a stock of 2.0mM NH_4Cl solution by dissolving 0.10698g in 1L of distilled deionised water. Using the stock solution, prepare various concentrations of NH_4Cl standards as follows:

NH_4Cl standard conc. (mM)	Volume of 2.0mM stock added (ml)	Volume of distilled water added (ml)
0	-	10.0
0.2	1.0	9.0
0.4	2.0	8.0
0.6	3.0	7.0
0.8	4.0	6.0
1.0	5.0	5.0
1.5	7.5	2.5
2.0	10.0	-

In 1ml cuvettes, add 350 μl of Reagent A of the ammonia detection assay. Add 100 μl of the standards and then add 350 μl of Reagent B of the ammonia detection assay in each cuvette. Leave for 15 minutes at room

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temperature and thereafter measure absorbance at 600nm, using a spectrophotometer.



B3. 50mM potassium phosphate buffer

0.1M K₂HPO₄ stock solution: 17.4g in 1L of distilled water

0.1M KH₂PO₄ stock solution: 13.6g in 1L of distilled water

Add desired volumes of both the stock solutions in 100ml distilled deionised water, as follows:

Desired pH	Volume of 0.1M K ₂ HPO ₄ added (ml)	Volume of 0.1M KH ₂ PO ₄ added (ml)
5.7	6.5	93.5
6.0	12.3	87.7
6.5	31.5	68.5
7.0	61.0	39.0
7.5	84.0	16.0
8.0	94.7	5.3

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B4. 50mM sodium acetate buffer

0.2M acetic acid stock (A): 11.55ml of glacial acetic acid in 100ml of distilled water.

0.2M sodium acetate stock (B): 16.4g sodium acetate in 1L of distilled water.

Add desired volumes of both stock solutions in 100ml of distilled deionised water, as follows:

Desired pH	Volume of A added (ml)	Volume B added (ml)
4.0	41.0	9.0
5.0	14.8	35.2

B5. 50mM *Tris*-HCl buffer

0.1M *Tris* stock solution: 12.114g of tris(hydroxymethyl) aminomethane in 1L of distilled deionised water.

Adjust 100ml of stock solution to desired pH using concentrated HCl and then make-up to 200ml using distilled water.

Appendix C: SDS-PAGE protocol

C1. Reagents

1. Resolving gel buffer (1 M, pH 8.8)

For 500ml:

<i>Tris</i>	60.6g
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HCl (concentrated)	7.3ml
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Dissolve in distilled water, check pH and adjust if necessary

2. Stacking gel buffer (1M, pH 6.8)

For 500 ml:

<i>Tris</i>	60.6g
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HCl (concentrated)	41.0ml
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Dissolve in distilled water, check pH and adjust if necessary

3. 30% Acrylamide Stock

For 250ml:

Acrylamide	75g
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<i>Bis</i> -Acrylamide	2g
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Dissolve in 250ml distilled water

4. Running buffer (stock solution)

For 1000ml:

<i>Tris</i>	30.3 g
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Glycine	144.1g
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SDS	10.0g
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Dissolve in 1L of distilled water

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5. Running buffer (working solution)

Dilute stock solution 10x with distilled water

6. Dissociation buffer

For 50ml:

SDS	5g
Mercaptoethanol	5.0ml
Glycerol	7.5ml
0.2% Bromophenol blue	2.5ml
<i>Tris</i> -HCl (1M, pH 6.8)	6.3ml
Distilled water	28.7ml

7. Staining solution

For 1000ml:

Methanol	450ml
Glacial acetic acid	100ml
Coomassie brilliant blue (R250)	2.0g
Distilled water	450ml

8. Destaining solution

For 1000ml:

Methanol	450ml
Glacial acetic acid	70ml
Water	480ml
Glycerol	100ml

C2. Preparation of the Gel

a. Plug

1. Mix stock solutions in the following order:
3ml of 30% acrylamide stock solution
80µl of 10 % ammonium persulphate (prepare fresh)
40µl of TEMED
2. Immediately place plug at the bottom of the gel plate with Pasteur pipette and allow setting.

b. 10% Resolving Gel

Mix stock solutions in the following order:

- 13.35ml of acrylamide stock solution
- 15.0ml of *Tris*-HCl buffer (pH 8.8)
- 9.25ml distilled water
- 400µl 10% SDS (omit and replace with water for Non-denaturing)
- 300µl of 10% APS (prepare fresh)
- 20µl of TEMED
- Pour resolving gel into gel plate with Pasteur pipette
- Pour a thin layer of water across the top of the gel and allow setting for approximately 30 minutes

c. 4% Stacking gel

Mix stock solutions in the following order:

- 2ml of the acrylamide stock solution
- 1.9ml *Tris*-HCl buffer (pH 6.8)
- 9.25ml distilled water
- 1.0ml of 80% glycerol

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100µl of 10% APS (prepare fresh)

150µl 10% SDS

20µl TEMED

-remove the water from the surface of the resolving gel and pour stacking gel with Pasteur pipette.

-add a 10-tooth comb and leave to set for about 20 minutes

-once set, place the gel in the base of the electrophoresis apparatus

-remove comb and fill apparatus with running buffer

C3. Sample loading

1. In an Eppendorf tube, mix 50µl of enzyme solution with 50µl of dissociation buffer. Heat the solution for 5 minutes at 100°C. Allow cooling to room temperature and load the sample into the gel.

C4. Electrophoresis

Electrophoresis at 100-120 V until the dye is within 1 cm of the bottom (3 hours)

C5. Staining and Destaining

Cover the gel with Coomassie staining solution for 30 to 60 minutes.

Destain for 2x for 20 minutes with destaining solution I.

Destain overnight with destaining solution II.